THE ROLE OF EXTRACELLULAR MATRIX PROTEINS IN TRAUMATIC BRAIN INJURY AND CELL TRANSPLANTATION

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LIST OF ABBREVIATIONS

TBI	traumatic brain injury
CSPGs	chondroitin sulphate proteoglycans
BBB	blood-brain barrier
TNF	tumor necrosis factor
NMDA	N-methyl-D-aspartate
CCI	controlled cortical impact
ECM	extracellular matrix
NSC	neural stem cells
FN	fibronectin
LN	laminin
pFN	plasma fibronectin
Bcl-2	B-cell leukemia/lymphoma 2
CNS	central nervous system
CnI, collagen I	collagen type I
GFP	green fluorescent protein
DMEM/F12	Dulbecco's modified eagle medium: nutrient mix F-12
bFGF	basic fibroblast growth factor
MWM	Morris water maze
PBS	phosphate buffered saline
OCT	optimal cutting temperature
GFAP	glial fibrillary acidic protein
NG2	neuron-glial antigen 2
PDGF	platelet-derived growth factor

CNPase	2′,3′-cyclic nucleotide 3′-phosphodiesterase
SD	standard deviation
WT	wild type
IV	intravenous
DNA	deoxyribonucleic acid
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TUNEL	terminal deoxynucleotidyl-transferase-mediated biotin-dUTP nick end labeling
PLL	poly-l-lysine

SUMMARY

With over 50,000 deaths and 80,000 disorders annually in the United States resulting from traumatic brain injury (TBI), there is a demand for improved therapeutic strategies. Cell transplantation offers the potential to treat TBI by targeting multiple mechanisms in a sustained fashion. However, efforts are needed to improve survival and integration of transplanted cells, and ultimately enhance functional recovery. Using tissue engineering strategies, we aimed to mimic key aspects of fetal tissue grafts by combining neural stem cells with a fibronectin or laminin based scaffold that could be delivered to the injured brain in a minimally invasive fashion. We found that the incorporation of extracellular matrix proteins into a cell transplantation paradigm led to improved donor cell survival and restored cognitive ability for treated animals. To begin to examine how fibronectin and laminin mediate these improvements, we first examined the endogenous role of these two proteins in the injured brain. Using a clinically-relevant model of TBI, we found both proteins are increased in the injured brain at acute time points. The spatial localization of fibronectin and laminin with specific support cells in the brain suggests a role for these proteins in repair, warranting further investigation. Using conditional plasma fibronectin knockout animals, we found that fibronectin is neuroprotective to the traumatically injured brain. Specifically, injured fibronectin knockout animals had more severe motor and cognitive deficits, increased cell death, and decreased retention of phagocytic cells compared to injured wild type animals. Thus, we have identified novel therapeutic treatments for TBI which utilize tissue engineered transplants and/or exploit endogenous repair mechanisms for fibronectin.

CHAPTER 1

INTRODUCTION

Traumatic Brain Injury

Traumatic brain injury (TBI) results from a sudden and external physical impact to the head, and often leads to motor (e.g., loss of ambulation, balance, coordination, fine motor skills, strength, and endurance) and cognitive (e.g., loss of communication, information processing, memory, and perceptual skills) impairment. Annually, there are over 50,000 deaths and 80,000 disabilities resulting from TBI in the United States alone (Langlios, 2004; Thurman et al., 1999). The economical impact, costing over $4.5 billion (in direct costs) per year (Max et al., 1991), as well as the health and sociological implications prompt the demand for clinically effective treatments.

The physical impact to the brain tissue initially causes necrotic cell death in the underlying tissue, followed by apoptotic cell death in surrounding tissue due to multiple subsequent events such as edema, ischemia, excitotoxicity, increase in free radicals, and altered gene expression (for review, see McIntosh et al., 1998 and/or Verma, 2000). Both primary and secondary insults initiate a glial response, which acutely acts to sequester and clean debris at the injury site. Cellular components of the glial scar include reactive astrocytes, which help buffer excess glutamate and secrete neurotrophic factors, and activated microglia, which along with monocyte-derived macrophages, clear out dead tissue. However, extracellular components of the glial scar that forms adjacent to the injury site have been found to inhibit neurite extension (e.g., chondroitin sulphate proteoglycans (CSPGs), Nogo protein), thus limiting regeneration (for review, see Properzi et al., 2003). It has also been appreciated recently that the brain may be attempting to repair through developmental processes, as evidenced by the increases in

neurogenesis and angiogenesis that occur following TBI (Dash et al., 2001; Kernie et al., 2001; Ramaswamy et al., 2005). The schematic in **Figure 1.1** summarizes these post-TBI events. This evolution of the brain injury response exemplifies the dynamic interplay between events promoting repair and regeneration competing with mechanisms of damage and inhibition. Ideal treatment strategies will exploit and complement native repair mechanisms while suppressing inhibitory mechanisms.

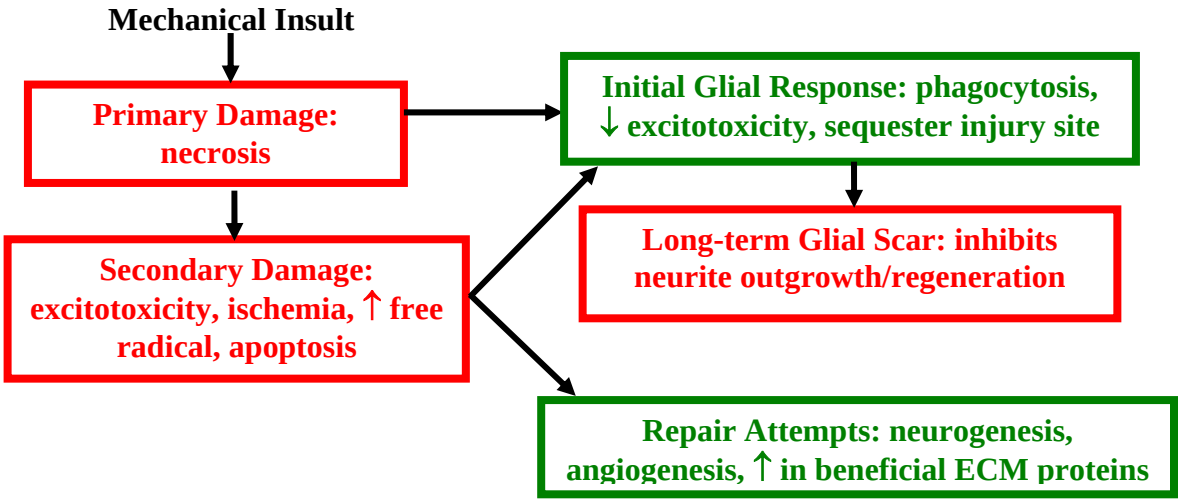

Figure 1.1: Schematic of events following focal traumatic brain injury

Mechanisms of damage and inhibition or regeneration (in red boxes) occur alongside permissive and reparative mechanisms (in green boxes). Better understanding mechanisms on both sides of the response can aid in the development of more effective clinical treatments for TBI.

Dual Role of Inflammation in Traumatic Brain Injury

While the brain is considered immune-privileged (due to the tight regulation of the CNS microenvironment which largely excludes blood leukocytes from entering), immune responses occur during pathological conditions (Perry et al., 1997). In TBI, the initial release of cell contents from the primary necrosis initiates an inflammatory reaction. While inflammation occurring in the brain is associated with deleterious events, the beneficial role of inflammation in TBI has gained appreciation (Correale and Villa, 2004; Lenzlinger et al., 2001; Morganti-Kossmann et al., 2002; Schwartz, 2000). The major events of inflammation following TBI are summarized here with an emphasis on the dual nature of this endogenous immune response.

The three major classes of protein mediators of inflammation are cytokines, chemokines, and complement proteins. Pro-inflammatory cytokines (e.g., tumor necrosis factor (TNF), interleukins-1 and -6) are released within minutes following TBI (Morganti-Kossmann et al., 2002). Studies with specific cytokine knockout mice, as well as in vitro studies indicate that these cytokines may have acute deleterious effects (e.g., blood-brain barrier (BBB) dysfunction, promotion of neuronal death), but are beneficial at later time points (e.g., induce synthesis of anti-inflammatory cytokines, induce neurotrophic factors, promote proliferation of oligodendrocyte precursor cells which may help in remyelination) (Morganti-Kossmann et al., 2002). Neurons and glial cells (astrocytes, microglia, and oligodendrocytes) can produce both chemokines (e.g., TNFα, interferon-γ) and complement proteins (e.g., C3, C5), and have receptors for these proteins. In a similar dual role, chemokines and complement proteins are involved in acute BBB dysfunction and edema, while these proteins eventually lead to increased nerve growth factor production in astrocytes and microglia. Furthermore, complement proteins have been found to protect neurons from excitotoxicity-induced apoptosis and promote opsonization (Schmidt et al., 2005).

With respect to the cellular aspect of the inflammatory response, microglia (resident brain immune cells) are the first to respond (minutes to hours) by proliferating, and becoming activated microglia which migrate to the area of injury, where they essentially function as macrophages (Kato and Walz, 2000; Ladeby et al., 2005). In a traumatic injury, which is associated with increased BBB permeability, leukocytes from the blood can pass through the endothelium at the site of injury; and this process is mediated by cytokines, chemokines, and complement proteins (Schmidt et al., 2005). Neutrophils are the first to infiltrate (hours to days), followed by monocytes (days) (Kato and Walz, 2000). Again, there are both beneficial and deleterious roles for these immune cells. The oxidative burst of neutrophils and macrophages is harmful because of the release of oxygen free radicals and neurotoxic enzymes (Schmidt et al., 2005), however, both activated microglia and monocyte-derived macrophages aid in clearing debris from dead/damaged cells via phagocytosis (Hauwel et al., 2005). Because of the dual nature of the inflammatory response, treatments for TBI that target specific cells or proteins involved in the inflammation response may not be ideal (McIntosh et al., 1998).

Clinical Treatments for Traumatic Brain Injury

Current treatment methods in clinical practice primarily aim to reduce intracranial pressure in an effort to minimize brain damage caused by swelling. Examples include moderate hypocapnia and mannitol (first line measures), followed by barbiturates, moderate hypothermia, or a decompressive craniectomy (second line measures) if early attempts fail (Sahuquillo and Arikan, 2006). However, these therapies have a modest effect on acute brain damage or subsequent cell death pathways which lead to functional impairment (Roberts et al., 1998). Furthermore, these treatments do not provide sustained efforts to promote repair or regeneration. Since the majority of TBI patients are young adults, there is a demand for chronic treatments that would prevent further brain

damage *and* help repair damage that has already occurred. Treatment approaches under investigation for TBI in the past several years aim to target one or more of the pathological events following TBI in an effort to rescue cells or promote repair and regeneration. While many prospective treatments seemed promising in animal models, results in clinical trials have been mixed at best. For example, excitotoxicity results from excess glutamate released from necrotic cells over stimulating neurons (primarily through NMDA receptors), causing increased intracellular calcium levels and ultimately cell death; and targeting excitotoxicity showed therapeutic potential in animal models. However, various treatments that mediate along this path, such as glutamate antagonists, were not found to be effective for humans with TBI (Ikonomidou and Turski, 2002; Willis et al., 2004). Similar results occurred with other investigational drugs including free radical scavengers and steroids (Narayan et al., 2002). These treatments may have failed in the clinic because they target pathways that are both deleterious and beneficial, thus the dosage and time of treatment are critical to not interfere with normal homeostasis or reparative mechanisms in the brain. Furthermore, these treatments targeted single mechanisms, which may not be enough in light of the multi-faceted pathology. Therapies that currently seem more promising, such as hypothermia or progesterone administration, address multiple pathological events.

Experimental Model – Controlled Cortical Impact Injury

The majority of traumatic injuries to the head occur in falls, followed by motor vehicle accidents, making blunt trauma by far the most prevalent type of TBI (Langlios, 2004). Researchers model this type of focal injury by impacting the exposed mammalian cortex, typically using one of the following experimental models (listed in chronological order of their development): weight drop, fluid percussion, or controlled cortical impact (CCI). The CCI injury, a controlled mechanical compression to the cortical surface produces consistent and reproducible injuries, has a low mortality rate compared to the other

models, and has a well-characterized pathophysiology that closely approximates events seen in human TBI, including acute subdural hematoma, BBB dysfunction, and edema (Cernak, 2005; Dixon et al., 1991; Morales et al., 2005). It has also been shown that using the CCI model in mice produces long-term motor and cognitive deficits that mimic certain aspects of clinical TBI (Fox et al., 1998; Shear et al., 2004). Using this model, the experimenter can control velocity, depth, duration, and angle of the impact; though the velocity and depth are the best predictors of injury severity (Cernak, 2005; Morales et al., 2005). An injury to the cortex, which impacts the mouse brain at a velocity of 6.0 m/sec and deforms the brain 1 mm is considered moderate, and is associated with both cortical and subcortical damage and produces sustained sensory/motor and spatial learning deficits (Fox et al., 1998). The CCI injury model in mice is utilized throughout this dissertation work, using a custom-built pneumatically-driven impactor device (see schematic in **Figure 1.2**) and with the following parameters to obtain moderate, unilateral injuries: velocity = 6.0 m/sec, depth = 1 mm below dura, duration in brain = 150 msec, and angle = 15° from the vertical.

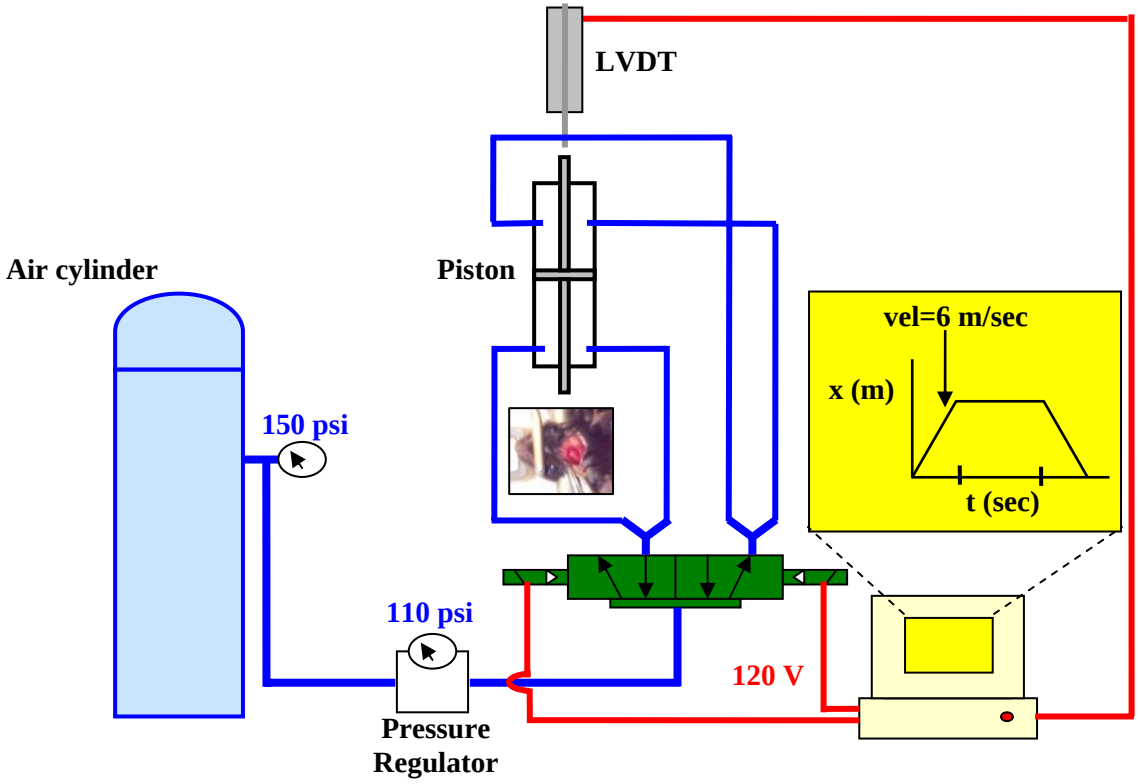

Figure 1.2: Schematic of pneumatically-driven piston used for controlled cortical impact injuries

Using the custom-built device depicted in this figure, a controlled impact is applied to the exposed rodent cortex. Air flow is represented by blue lines, and red lines symbolize electrical transmission. Briefly, compressed breathable air, regulated to 110 psi, moves through one of two ports in the double solenoid valve (shown in green). Air leaving one side will push air into the top of the piston, driving the piston down, while the other side has the opposing affect. The switch between the two movements is controlled via LabVIEW software (National Instruments, Austin, TX), which also reads from a linear variable displacement transducer connected in series with the piston to measure velocity and duration of the impact.

Relevant Extracellular Matrix Proteins Involved in TBI Response

The levels of various extracellular matrix (ECM) proteins increase following TBI. We are interested in investigating those proteins that may be involved in repair or regeneration. Furthermore, we are interested in ECM proteins which may be exploited for use in tissue engineered constructs containing neural stem cells (NSC) in an effort to recapitulate fetal-like tissue for transplantation after TBI. Both fibronectin (FN) and laminin (LN) play a major role in neural development and are found in increased levels following injury, where they potentially play a reparative role. Thus, these proteins are the focus of this dissertation work.

Fibronectin

Fibronectin, a large (~440kDa) glycoprotein, is a common adhesive protein of the interstitial matrix (for review, see (Pankov and Yamada, 2002)). There are 20 different FN isoforms in humans, generated by alternative splicing of a single *FN* gene. The majority of the splicing patterns are cell-type specific, and give rise to a number of different cellular FN isoforms, which are found immobilized in ECM. Another type of FN is plasma FN (pFN), which is primarily made by hepatocytes and secreted as a soluble form into the blood plasma (Owens and Cimino, 1982).

Fibronectin interacts with cells primarily via integrin receptors, and this binding signals a sequence of events including changes in gene expression, cell survival, proliferation, differentiation, and migration (for review, see (Danen and Yamada, 2001)). While many integrins have been shown to bind FN, including $\alpha_3\beta_1$, $\alpha_4\beta_1$, $\alpha_8\beta_1$, $\alpha_{IIb}\beta_3$, $\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$ and $\alpha_v\beta_6$, the classic FN-binding integrin is $\alpha_5\beta_1$ (Sonnenberg, 1993). Fibronectin predominately binds to the $\alpha_5\beta_1$ integrin (as well as others) via the Arg-Gly-Asp (RGD) amino acid sequence located in the central binding domain, and this is synergistically enhanced by binding to the Pro-His-Ser-Arg-Asn (PHSRN) sequence (Aota et al., 1994).

The binding of FN to $\alpha_5\beta_1$ promotes cell adhesion and triggers events such as cell migration and signal transduction (Akiyama, 1996).

Fibronectin in the Normal and Injured Brain

Fibronectin is crucial for embryogenesis (George et al., 1993), and is involved in the developing nervous system (Reichardt and Tomaselli, 1991). Fibronectin promotes both the survival and migration of neural crest cells (Henderson and Copp, 1997; Testaz and Duband, 2001), and also supports migration of neurons to different cortical regions (Sheppard et al., 1995). Furthermore, FN promotes neurite extension and synapse formation in the developing brain (Einheber et al., 1996). While FN is largely absent in the adult brain (Bellail et al., 2004; Maenpaa et al., 1997), levels increase following brain injury. Human TBI studies have shown an increase in FN post-injury (Liu and Sturner, 1988), and an increase in FN has also been seen in various brain injury models including penetrating stab wound (Egan and Vijayan, 1991; Mizutani and Kimura, 1987; Pasinetti et al., 1993), cortical cold injury (Nag et al., 1997; Suzuki and Choi, 1990), kainic acid lesion (Hertel et al., 2000). Since the elevated FN levels after TBI are largely due to an increase in BBB permeability (Nag et al., 1997) and cellular FN is not expressed in wounded tissue following ischemic brain injury (Sakai et al., 2001), it is hypothesized that the majority of FN expression after brain injury is plasma borne.

It has been suggested that FN plays a role in repair following TBI (Egan and Vijayan, 1991; Liu and Sturner, 1988; Nag et al., 1997), though this has not been experimentally verified. However, following cerebral ischemia, Sakai et al. (2001) showed that pFN null mice have greater lesion size, increased number of apoptotic cells, and decreased Bcl-2 expression, indicating that pFN is neuroprotective in brain ischemia. Furthermore, exogenous functional FN peptides have also been shown to reduce polymorphic

leukocyte accumulation, infarct size and improve neurological outcome when administered intravenously for experimental cerebral ischemia (Yanaka et al., 1996).

Fibronectin in Wound Repair and Phagocytosis

Fibronectin has been extensively studied in peripheral wound healing, and pFN is a potent stimulator of the wound healing process (Lariviere et al., 2003). The two major roles for FN in would healing are in the formation of blood clots (both as a scaffold for other matrix proteins and as a cell attractant) and opsonization, which is the process by which foreign bodies are made more amenable to phagocytosis (for review, see (Clark, 1988)). For the purposes of the dissertation work presented here, we will focus on the latter. Fibronectin is a well-known opsonin and mediates removal of several types of foreign bacteria (Mosser, 1994; Shinji et al., 2003). Furthermore, FN also opsonizes tissue debris for removal by phagocytes (Martin et al., 1988). Following most types of trauma, pFN levels are depressed due to the binding of pFN to the gelatinous injured tissue (La Celle et al., 1990; Martin et al., 1988; Reese et al., 1982). In addition to its opsonic functions at the site of injury, FN aids in macrophage recruitment to this injured tissue. Fibronectin fragments are chemoattractants for monocytes (Proctor, 1987), and FN enhances adhesion and migration of monocytes via β1 integrin binding (Kao et al., 2001; Meng and Lowell, 1998). Upon entering the site of injured tissue, monocytes differentiate into macrophages, which correlates with increased expression of FN integrin receptors (Akimov and Belkin, 2001; Mograbi et al., 1999). All of these findings indicate FN is a critical player in the inflammatory response with respect to phagocytosis of injured tissue.

More specific to brain injury, FN regulates microglial cell behavior. Because the brain is protected by the tight junctions of the BBB, leukocytes from the blood typically cannot enter (Perry et al., 1997). Microglia are the resident immune cells available to respond

quickly in the immune-privileged brain. As microglia become activated (taking on more of a phagocytic phenotype), FN integrin expression increases (Milner and Campbell, 2003). Binding to FN promotes microglial cell proliferation, adhesion and migration (Liao et al., 2005; Nasu-Tada et al., 2005), all of which are involved in recruitment of microglia to injured brain regions. Fibronectin binding also increases microglial secretion of nerve growth factor and transforming growth factor-beta (Liao et al., 2005), both of which have beneficial functions in the injured brain (Lenzlinger et al., 2001; Sinson et al., 1997).

Fibronectin and Apoptosis

Anoikis refers to increased apoptosis in response to the absence of cell-ECM interactions. Fibronectin has been shown to be anti-apoptotic in various in vitro cell systems including umbilical vein endothelial cells (Fukai et al., 1998), Chinese hamster ovaries (Matter and Ruoslahti, 2001; Zhang et al., 1995), and prostate cancer cells (Morgan et al., 2000). More specific to the nervous system, Gibson et al. (2005) showed that FN is anti-apoptotic for Ntera2 neurons in vitro. In all cases, the reduction in cellular apoptosis was dependent on FN binding via the $\alpha_5\beta_1$ integrin. Furthermore, Fukai et al. (1998), Matter and Ruoslahti (2001), and Zhange et al. (1995) showed that FN-$\alpha_5\beta_1$ integrin binding causes increased expression of the anti-apoptotic molecule, Bcl-2. While Bcl-2 levels were not examined in the in vitro study of neuronal apoptosis, Sakai et al. (2001) reported that mice deficient in pFN have both increased apoptosis and decreased Bcl-2 expression following cerebral ischemia. In TBI, decreases in Bcl-2 precede apoptosis in TBI (Raghupathi et al., 2002) and overexpression of Bcl-2 has been found to be neuroprotective (Nakamura et al., 1999), indicating that FN binding may mediate Bcl-2 signaling in injured neural cells.

Laminin

Laminin, a trimeric glycoprotein ranging from 500-1000 kDa, is the major adhesive protein of the basal lamina (Colognato and Yurchenco, 2000). There are 16 known LN isoforms (12 found in mammals), made from various combinations of the five α, three β, and three γ subunits (Suzuki et al., 2005). The LNs are named (LN-1 to 16) based on the specific combination of these subunits. Recently, a simplified trimer nomenclature was proposed to name the LNs using the numbers associated with each subunit (Aumailley et al., 2005), thus both names are provided in the text below. The most studied cellular receptors for LNs are integrins, including $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_6\beta_1$, $\alpha_7\beta_1$, $\alpha_6\beta_4$; but other receptors include α-dystroglycan, and syndecans (Suzuki et al., 2005).

Laminin in the Normal and Injured Brain

Laminin plays an important role in neural development (Reichardt and Tomaselli, 1991). Laminin-1 (a.k.a. laminin-111), the primary LN type in early embryogenesis, promotes cell survival and migration in the developing nervous system (Liesi, 1992; Perris and Perissinotto, 2000), and both LN-1 and LN-2 (a.k.a. laminin-211) promote neurite outgrowth (Colognato and Yurchenco, 2000). In the mature brain, LN-10 (a.k.a. laminin-511) is found in the hippocampus and protects hippocampal neurons from excitotoxic lesions (Chen et al., 2003; Indyk et al., 2003), and laminin α2 subunit (likely in LN-2) is found in close association with dendritic spines in the hippocampus, and may be involved in synaptogenesis (Tian et al., 1997). Laminin-5 (a.k.a. laminin-332) is a major component of the ECM of the BBB, where it can be found in the basement membrane of blood vessels (along with LN-1 (Hagg et al., 1997)) and interacts with integrins on both endothelial cells and astrocytes (Wagner and Gardner, 2000). There is an increase in LN levels (either LN-1 or not specified) in the adult brain following clinical brain injury (Hausmann and Betz, 2000) and in experimental injuries, including cortical cold injury

(Kakinuma et al., 1998; Suzuki and Choi, 1990) and penetrating stab wound (Szabo and Kalman, 2004).

While the role of this increase in LN levels is unknown, it is thought to be reparative in nature (Kakinuma et al., 1998), based on the supportive functions of LN in development. Laminin has also previously been exploited as a treatment for brain injury. Functional LN peptides administered intravenously after cerebral ischemia led to reduced polymorphic leukocyte accumulation and infarct size and improved neurological outcome (Yanaka et al., 1997). Furthermore, LN-based hydrogels implanted into a brain cavity encouraged cell infiltration and angiogenesis and reduced glial scar formation (Hou et al., 2005). The use of LN as treatment provides further evidence for a beneficial role after brain injury.

Neural Transplantation and Tissue Engineering

Evidence suggests that cells in the CNS can regenerate if they are in the proper environment (David and Aguayo, 1981). Transplanting cells may help approximate this environment and mediate repair and recovery through trophic support, cell-cell contact induced signaling, and/or replacing cells lost to injury.

Fetal Tissue Transplantation

Transplantation of fetal tissue has been shown to promote recovery of central nervous system (CNS) injury as well as neurodegenerative diseases (for review, see Koutouzis et al., 1994; Subramanian, 2001). Specific to brain injury, fetal tissue grafts have been shown to improve functional recovery following various models of TBI. For example, fetal frontal cortical tissue transplants promoted cognitive recovery following frontal cortical lesions (Labbe et al., 1983). Also, fetal cortical tissue transplanted following fluid percussion injury led to improved motor and cognitive recovery (Sinson et al.,

1996). Unfortunately, there are some limitations to the clinical use of fetal tissue transplants (for review, see Stein, 1991 and/or Stein and Glasier, 1995). There are both ethical and availability issues with using fetal sources. From a technical standpoint, tissue pieces are difficult to keep viable in vitro, making an "off-the-shelf" treatment unlikely. In addition, intracranial injection of tissue pieces requires potentially invasive surgical procedures. Based on these considerations, transplantation of cells from other sources offers a promising alternative.

Cell Transplantation

Transplantation of cells in suspension may overcome many of the limitations associated with using fetal tissue, and thus may be more clinically feasible. For example, transplantation of cells offers a potentially renewable treatment source and surgical procedures may be less invasive compared to transplanting tissue pieces. In particular, stem cells are an attractive transplantation candidate due to their ability to proliferate extensively in vitro and their multipotential characteristics. Previous work in our laboratory has determined that transplanting NSC into the ipsilateral striatum after TBI results in sustained motor and cognitive improvement (Shear et al., 2004). There are numerous other investigations into cell transplantation paradigms for TBI with differing cell types and delivery times / locations with varying responses of donor cell function and effects on host recovery (for review, see Schouten et al., 2004). In addition to investigating these factors, the method of delivery must be considered, as a common problem with cell transplants is their limited survival and interaction with host cells, particularly compared to fetal tissue grafts. Studies that directly compare cells in suspension and tissue pieces have shown that donor cells transplanted in the tissue pieces had significantly improved survival; and furthermore, animals receiving the tissue pieces had significantly better functional outcome compared to those treated with cell suspension grafts (Clarkson et al., 1998; Hoovler and Wrathall, 1991; Sinson et al.,

1996). This enhanced donor cell survival for transplantations of intact tissue may be due to the presence of three-dimensional structure and a higher accessibility of extracellular adhesive proteins for the donor cells to attach. Cell transplantation has already shown promise in the clinic for treating severe TBI (Seledtsov et al., 2005), and it is important to move cell transplantation research towards improving survival and integration of donor cells in an effort to further advance cell transplantation therapy.

Neural Stem Cells

Stem cells are receiving attention as candidate cells for transplantation, due largely to the proliferative and pluri-/multipotent nature of these cells. The fate of these cells is dictated by both in vitro preparation and the host environment. This is important because multipotent stem cells can adapt to the "needs" of the host tissue (Yandava et al., 1999). Neural stem cells are multipotent stem cells that have the capacity to differentiate into the major cells in the CNS: neurons, astrocytes, and oligodendrocytes, and have many potential applications in CNS transplantation. Neural stem cells persist in the adult brain (Johansson et al., 1999; Rietze et al., 2001) and contribute to neurogenesis that occurs throughout adult mammalian life in the olfactory and hippocampal regions (Dash et al., 2001; Johansson et al., 1999). Furthermore, the rate of neuro- and gliogenesis increases following injury (Dash et al., 2001; Johansson et al., 1999; Kernie et al., 2001; Ramaswamy et al., 2005). This is thought to be an attempt at self-repair and plasticity, but regeneration in the brain is limited due to mechanisms that are not completely understood, but are attributed to an inhibitory environment. Transplanting NSC into the injured brain may augment the neuro- and gliogenic environment that the brain inherently attempts to create following injury.

Neural Stem Cells and Fibronectin and Laminin

In the developing nervous system, NSC utilize both FN and LN for survival and migration signals (Henderson and Copp, 1997; Testaz and Duband, 2001). For example, previous work in our laboratory examined the behavior of NSC in vitro, and found that adhesion and migration on both FN and LN is significantly higher than other ECM proteins, including collagen types I and IV, and CSPG (Tate et al., 2004). Furthermore, we showed that these behaviors are primarily mediated through $\alpha_5\beta_1$ and $\alpha_6\beta_1$ integrin binding to FN and LN, respectively (Tate et al., 2004). In addition, β_1 integrins are also critical to NSC survival and proliferation (Leone et al., 2005). Based on this in vitro evidence and the roles of FN and LN in development, transplanting NSC with FN or LN may aid in survival and integration of these cells in the injured brain.

Neural Tissue Engineering in the Brain

Tissue engineering offers the ability to repair or replace lost tissue using a multi-faceted approach. The classic description of a tissue engineered construct is the combination of cells with an appropriate scaffold that provides both mechanical and chemical support. The addition of the cellular component can occur ex vivo or the scaffold can recruit cells in the body. Specific combinations of cells and scaffolds are designed to meet the needs of the physiologic and pathologic system of interest. To date, there have been several efforts to utilize tissue engineering approaches in the nervous system. Focusing on the brain, tissue engineering approaches have ranged from implanting encapsulated nerve growth factor secreting cells to treat Alzheimer's disease (Winn et al., 1996) to implanting NSC within porous polyglycolic acid scaffolds to treat hypoxia-ischemia (Park et al., 2002).

We are developing a construct containing NSC and a bioactive scaffold utilizing ECM proteins that can be delivered in a minimally invasive fashion, for the purpose of

increasing donor cell survival and integration within the host tissue, thereby improving repair and recovery. We have shown that there is increased donor cell migration when NSC are transplanted within a FN/collagen I (FN/CnI) scaffold compared to NSC transplanted alone after TBI (Tate et al., 2002). This increased migration to areas of secondary insult (e.g., fimbria-fornix in the hippocampus) implies these cells are responding to cues of the injured brain, indicating that delivery of NSC with specific ECM proteins improves integration with the host tissue. Moreover, we can infer that the enhanced migration is due to the presence of FN in the scaffold since these cells have increased in vitro adhesion and migration on FN, but not on CnI (compared to tissue culture plastic) (Tate et al., 2004). Utilization of appropriate ECM proteins in cell transplantation paradigms may be crucial to increase donor cell survival and integration, ultimately leading to improved repair of the injured and diseased CNS.

CHAPTER 2

LAMININ AND FIBRONECTIN ENHANCE SURVIVAL OF NEURAL STEM CELLS TRANSPLANTED INTO THE INJURED BRAIN, RESULTING IN FUNCTIONAL RECOVERY

Abstract

Cell transplantation offers the potential to treat central nervous system injuries, largely because multiple mechanisms can be targeted in a sustained fashion. It is crucial that cells are transplanted in an environment that is favorable for their extended survival and integration with the host tissue. Given the success of using fetal tissue grafts for traumatic brain injury, it may be beneficial to mimic key aspects of this tissue (e.g., three-dimensionality, extracellular matrix support) to deliver cells. Extracellular matrix proteins such as fibronectin and laminin are involved in neural development and may provide adhesive support for donor cells and mediate cell signaling events. In this study, neural stem cells were transplanted into the traumatically injured mouse brain within a tissue engineered construct containing either a laminin- or fibronectin-based scaffold. Behavioral results indicated that mice receiving neural stem cells within the laminin-based scaffold perform better than untreated mice on the Morris water maze task. Furthermore, by 8 weeks post-transplant, cells delivered within the fibronectin-based scaffold showed improved survival compared to those transplanted in media alone, and this was more exaggerated for cells delivered within the laminin-based scaffold, which correlates with the behavioral results. Initial delivery conditions are an important consideration, and the use of appropriate extracellular matrix based scaffolds can be exploited to improve cell transplantation therapy.

Introduction

Traumatic brain injury (TBI) remains a major health problem with over 1.4 million new cases of TBI annually in the United States alone (Langlios, 2004), however there is currently no clinical treatment available that aids in repair or regeneration. Considering the complex and dynamic milieu of the injured brain, cell transplantation is an attractive treatment option because multiple therapeutic mechanisms can be targeted in a sustained fashion. Moreover, cells, particularly stem cells, are adaptable to their environment and can communicate with the injured brain cells. The robust and plastic nature of stem cells makes them favorable candidates in cell transplantation paradigms. We are interested in utilizing neural stem cells (NSC), which are multipotential cells capable of differentiating into the major cell types along the neural lineage, specifically, neurons, astrocytes, and oligodendrocytes.

One important consideration for cell transplantation in the central nervous system (CNS) is the preparation and delivery of transplants. Studies have shown that transplantation of tissue pieces have better graft survival and lead to improved behavioral recovery compared to cell suspension grafts (Clarkson et al., 1998; Hoovler and Wrathall, 1991). For TBI, transplanting fetal tissue pieces to the injured brain has been shown to significantly improve behavioral recovery (Labbe et al., 1983; Sinson et al., 1996). However, delivery of intact tissue is more invasive than injections of cell suspensions. Furthermore, ethical considerations and availability issues associated with transplanting fetal tissue prompt the demand to improve cell suspension transplants. Many aspects of the intact tissue may contribute to the success of this treatment including three-dimensionality, cell-cell support (with multiple cell types), growth factor cues, and cell-matrix support. Using tissue engineering, a subset of these features can be recapitulated. In this study, we examined augmenting delivery of NSC with an extracellular matrix (ECM) protein-based scaffold support. The two ECM proteins that we examined were

fibronectin (FN) and laminin (LN). Both of these glycoproteins are critical to neural development and have been found to promote cell survival and migration, neurite outgrowth and synapse formation (Colognato and Yurchenco, 2000; Perris and Perissinotto, 2000; Testaz and Duband, 2001). In vitro, NSC have increased adhesion and migration on both FN and LN compared to collagen types I or IV or tissue culture plastic, and this behavior was mediated by β_1 integrins present on the NSC (Tate et al., 2004). Thus, FN and LN can provide both a physical support and initiate appropriate cell signaling to improve survival and integration of donor cells in the injured brain. Moreover, the use of an appropriate thermally-reversible protein or polymer, such as collagen type I (CnI), allows for minimally invasive delivery of a construct which forms a three-dimensional gel in situ. We have previously shown that delivering NSC within a FN/CnI scaffold improved donor cell migration and survival at 3 weeks post-transplant (Tate et al., 2002). In the current study, we delivered NSC within a tissue engineered construct containing either FN/CnI or LN/CnI following controlled cortical impact (CCI) injury to assess the effects on both NSC function and cognitive behavior of treated animals.

Methods

Neural Stem Cell Isolation and Culture

Primary fetal-derived green fluorescent protein positive (GFP^+) NSC were used for this study. Pregnant transgenic B6-TgN(β-act-EGFP)osbY01 mice (C57BL6 background) were sacrificed by cervical dislocation, and embryos were isolated at gestational day 14.5 by Caesarian section. The skull layer was removed, allowing parasagittal cuts to be made in each hemisphere exposing the germinal layer. The ganglionic eminence of the germinal zone was then separated from the underlying tissue and mechanically dissociated into a single cell solution in Hank's balanced salt solution. Cells were cultured as spheres in media consisting of Dulbecco's modified eagle medium: nutrient

mixture F-12 (D-MEM/F12, 1:1; Invitrogen, Carlsbad, CA) containing insulin (25 μg/ml), transferrin (100 μg/ml), putrescine (60 μM), sodium selenite (30 nM), progesterone (20 nM), and glucose (0.3%). Human recombinant basic fibroblast growth factor (bFGF; 20 ng/ml; PeproTech, Rocky Hill, NJ) was added to the media every 2-3 days to keep the cells proliferating and clustered as neurospheres. Cells were passaged every 7-10 days, as dictated by increases in cell density. All cell culture reagents from Sigma-Aldrich (St. Louis, MO) unless specified otherwise. Experiments were performed using NSC at passage 3, and a total of 10 different flasks were used (from 5 different harvests). No correlation was detected between the flask used and the numbers of surviving cells at 1, 3 or 8 weeks post-transplant (**Figure 2.1**).

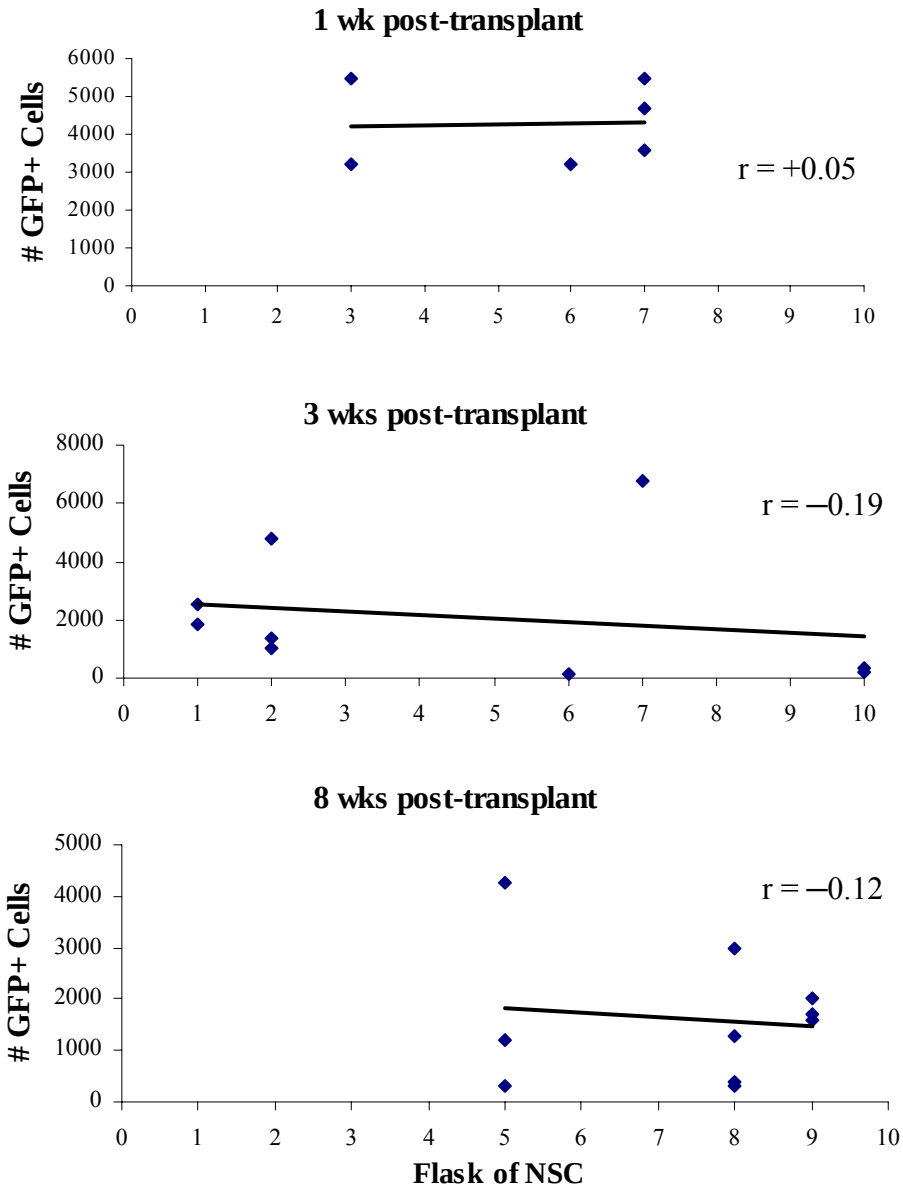

Figure 2.1: Correlation of donor cell survival with the flask of NSC used

Ten different flasks containing NSC were used throughout these experiments. Based on r-values close to 0 ($|r| < 0.2$), we determine there was not a correlation between the flask of NSC used in the transplant the number of surviving donor cells at 1, 3, or 8 weeks post-transplant.

Rheology Assessment of Collagen I Scaffolds

Rheological measurements were made to verify that the CnI-based scaffolds used in this study were gelling prior to reaching physiologic temperature. Scaffolds investigated were FN/CnI (final concentrations of 100 μg/ml and 1 mg/ml for FN (Invitrogen) and CnI (BD Sciences, Bedford, MA), respectively) and LN/CnI (final concentrations of 100 μg/ml and 1 mg/ml for LN (Sigma-Aldrich) and CnI, respectively). Samples (n=2 per scaffold) were analyzed on a Bohlin CVO rheometer (Bohlin, East Brunswick, NJ) using a parallel plate configuration. A temperature sweep from 15-45ºC (0.5º/sec) was performed at a constant frequency (0.045 Hz) and shear stress (0.221 Pa). Storage (G′) and loss (G″) moduli, and strain (γ) were plotted with respect to temperature to determine the characteristic temperature at which the solution transitions to form a gel (gelation temperature).

Controlled Cortical Impact Injury

Adult male C57BL6 mice (7-10 weeks old; Jackson Laboratory, Bar Harbor, MN) were anesthetized with 3% isoflurane and placed in a stereotaxic frame, where anesthesia was maintained (via gas mask) with 1-2% isoflurane. After exposing the skull, a 4 mm craniectomy was performed over the left fronto-parietal cortex, with the center at 2 mm posterior to bregma and 2 mm lateral to the midline. The CCI was produced with a 3 mm diameter pneumatically-operated metal impactor which entered the brain at a velocity of 6.0 m/sec. The impact to the brain occurred at a depth of 1.0 mm below the dura matter layer and the impactor remained in the brain for 150 msec. The impactor rod was angled 15° to the vertical to be perpendicular to the tangential plane of the brain curvature at the impact surface. A linear variable displacement transducer connected to the impactor recorded velocity and duration to verify the consistency of the injuries. After the CCI, the incision was sutured and animals were placed on warm pads for recovery. Sham injury surgeries consisted of anesthesia, skull exposure, craniectomy, and suturing. All mice were housed in a temperature-controlled environment with a 12:12-hr light-dark

cycle with food and water available ad libitum. All procedures conformed to guidelines set forth in the Guide for the Care and Use of Laboratory Animals and were approved by the Emory University Institutional Animal Care and Use Committee.

Transplantation Surgery

Transplant surgeries were performed 7 days after the CCI injury, because initial cell death mechanisms have subsided enough to provide a relatively stable environment and cavity formation (Hovda et al., 1995). All animals were re-anesthetized with 3% isoflurane and placed in a stereotaxic frame, where anesthesia was maintained (via gas mask) with 1-2% isoflurane. The previous incision was cut to expose the location of the impact. Transplants were delivered into the injury penumbra by placing a 10 μl syringe with 27-gauge needle (Hamilton, Reno, NV) in the center of 4 mm circle formed by the craniectomy, and 1.1 mm below the dura matter layer (thus 0.1 mm below the initial impact depth). All transplant injections (7 μl) were given at a constant rate over a 10-minute period using a syringe pump (Thermo Electron, Waltham, MA). After the injection, the needle was left in the brain for 5 minutes and removed slowly to minimize back flow through the needle track. Following this, the incision was sutured and animals placed on warm pads for recovery. Surgeries for the animals that had received sham injuries included the anesthesia, re-opening of the incision, and suturing.

Transplants of NSC Only

Green fluorescent protein positive NSC, injected at a density of ~30,000 cells/μL, were used in the transplant to easily distinguish between host and donor cells during histological analysis. Cell only transplants were at a 1:1 ratio of NSC to media (without bFGF). Vehicle transplants contained media (without bFGF) to control for the effects of the media components.

Transplants of NSC within ECM-based Scaffold

In an effort to enhance the presentation and binding of FN or LN to the donor NSC, these proteins were immobilized via binding to CnI. Cells with scaffold injections were at a 1:1 ratio of NSC to the FN/CnI or LN/CnI mixture to yield final concentrations of 100 μg/ml and 1 mg/ml for FN or LN and CnI, respectively. Scaffold only injections contained 1:1 media (without bFGF) to FN-CnI mix or LN-CnI mix.

Cognitive Behavior Testing

The Morris water maze (MWM) task was used to assess spatial learning performance (Fox et al., 1998; Morris, 1984). The MWM apparatus consisted of a white, circular tank (diameter = 1 m) filled with opaque water (20±1°C; nontoxic white tempura paint) to a depth of 64 cm (23 cm from the top of the tank). A platform was submerged to a depth of 1 cm and placed 28 cm from the wall of the pool in the center of the northeast quadrant. The position of the platform remained constant throughout the study. Each animal was given 2 trials per day with a 30 minute inter-trial interval for up to 12 consecutive days. At the start of each trial, the mouse was placed in the pool (facing the pool wall) at one of four randomly determined starting positions ("N, S, E, W"). Each mouse was allowed to swim freely in the pool until it found the hidden platform or until 60 seconds had elapsed. If the mouse did not find the platform in 60 seconds, he was manually guided to the platform, where mice remained for 15 seconds before being removed from the pool. A probe-trial using a visible platform to assess visuomotor abilities was conducted following the last MWM trial. A video tracking system (San Diego Instruments, San Diego, CA) was utilized to assess the latency to the platform. Two criteria were used to exclude mice from analysis: 1- mice that did not find the visual platform in under 55 seconds (to account for visual, motor, or motivation aspects of locating the platform), and 2- mice that did not have a single GFP^+ cell in their brains at 8 weeks post-transplant (to control for technical problems at the time of transplantation).

The following groups were compared, where the n-value denotes number of animals in each group that passed the two criteria listed above: 1- injured with NSC in media (n=5), 2- injured with NSC plus LN/CnI construct (n=5), 3- injured with NSC plus FN/CnI construct (n=4), 4- sham (n=10), 5- injured without treatment (n=10), 6- injured with media only (n=5), 7- injured with LN/CnI scaffold only (n=3), and 8- injured with FN/CnI scaffold only (n=6).

Histology

Mice were sacrificed for analysis at the following times post-transplant: 1 week (n=6 per treatment group), 3 weeks (n=7-8 per treatment group), and 8 weeks (n=8-10 per treatment group). Mice were re-anesthetized with an intraperitoneal injection of pentobarbital (80mg/kg), then perfused with 0.1M phosphate buffered saline (PBS), followed by 4% paraformaldehyde. The brains were harvested, immersed overnight in 4% paraformaldehyde at 4°C, then cryoprotected with 30% sucrose. The brains were then embedded in optimal cutting temperature (OCT) medium (Sakura, Torrence, CA), frozen, and stored at -80°C. A cryostat (Richard-Allan Scientific, Kalamazoo, MI) was utilized to serially section the brain into 10 μm thick coronal sections mounted onto gelatin-coated slides.

Assessment of Cell Survival and Migration

The number of surviving donor cells was estimated by quantifying GFP^+ cells found in the brains. We first confirmed that the constitutive fluorescence expression matched what was labeled using an antibody to GFP (1:500, A11122, Invitrogen) for each of the three time points examined (data not shown). Thus, we were able to count cells in unstained tissue. The optical fractionator method (Gundersen, 1986; Sterio, 1984) was utilized to estimate the number of GFP^+ cells by counting the entire area of interest for 40-80 sections at 40 μm increments per brain. For evaluation of migration, assessments

about the anatomical location of the cells and their radial dispersion from the initial cluster/clump of transplanted cells were noted.

Qualitative Assessment of Phenotype and Proliferation

Immunohistochemical analyses were employed to identify phenotypic markers on donor cells, and to identify if donor cells were undergoing proliferation at the time of sacrifice (n=2 per treatment group per time point; stained sections spaced 160 or 320 μm apart). Briefly, sections were rinsed in PBS, and then blotted and permeabilized in 20% goat serum and 0.5% tritonX-100 in PBS for 2 hr at room temperature, followed by 1 hr at 4°C. Sections were then incubated overnight at 4°C with one the following antibodies from Chemicon (Temecula, CA) unless noted otherwise: nestin (marker of neural progenitor cells; MAB377, 1:100), Sox-1 (marker of neural progenitor cells; AB5768, 1:250), glial fibrillary acidic protein (GFAP; marker of reactive astrocytes; MAP360, 1:400), β-tubulin III (marker of immature neurons; PRB-435P, 1:2000; Covance, Berkeley, CA), NeuN (nucleic marker of neurons; MAB377, 1:100), neuron-glial antigen 2 (NG2; marker of oligodendrocyte progenitor cells; AB5320, 1:100), platelet-derived growth factor (PDGF) receptor α (marker of oligodendrocyte progenitor cells; CBL 1366, 1:50), O4 (marker of immature oligodendrocytes; MAB345, 1:100), 2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNPase; marker of oligodendrocytes; MAB326, 1:100), or phospho-histone H3 (mitosis marker; #06-570, 1:100; Upstate, Charlottesville, VA). Following rinsing in PBS, sections were incubated with the appropriate species secondary conjugated to Alexa fluorophore 568 (in the red spectrum; Invitrogen) for 1 hour at room temperature. The percent of cells expressing specific phenotypic markers or H3 compared to the total number of GFP^+ cells was determined.

Statistics

A general linear model ANOVA, followed by Tukey's pairwise comparisons (Minitab, State College, PA) was used to compare the following groups for each time point: 1- injured with NSC in media ("NSC only"), 2- injured with NSC plus LN/CnI construct ("NSC+LN/CnI"), 3- injured with NSC plus FN/CnI construct ("NSC+FN/CnI"), 4- sham (for behavior study only), 5- injured without treatment (for behavior study only), 6- injured with media only (for behavior study only), 7- injured with LN/CnI scaffold only (for behavior study only), and 8- injured with FN/CnI scaffold only (for behavior study only). An alpha value of 0.05 was used to assess if the means were significantly different. Data are reported in the text as mean ± SD.

Results

Using collagen I scaffolds allows for minimally invasive delivery

Collagen I is thermally reversible, thus becomes more viscous with increasing temperature (at constant pH). Mixing bioactive matrix proteins with CnI enables minimally invasive delivery of a scaffold support for donor cells. The gelation temperature was assessed for both the FN/CnI and LN/CnI scaffolds to verify that a gel formed after injection, but prior to reaching physiologic temperature. **Figure 2.2** shows the rheological behavior as a function of temperature for the two scaffolds. The transition from a liquid to a gel is marked by the rapid increase in both the storage (G′) and loss (G″) moduli (Kobayashi et al., 1999). In addition, the strain (γ) rises slightly followed by a sudden drop, which is another hallmark of gelation (Takeuchi et al., 2003). When FN is bound to CnI to yield final concentrations of 100 μg/ml and 1 mg/ml, respectively, the mixture forms a gel at 27.8±1.0ºC (**Figure 2.2-top**). When LN is bound to CnI to yield final concentrations of 100 μg/ml and 1 mg/ml, respectively, the mixture forms a gel at 34.1±0.9 ºC (**Figure 2.2-bottom**). The temperature of a mouse brain under isoflurane anesthesia is 34.8±1.6°C (unpublished observations), thus, the rheology data

confirms that both the FN/CnI and LN/CnI scaffolds used in this study remained as a liquid when kept cool prior to transplantation, and formed a gel upon approaching physiologic temperature. Consequently, binding FN or LN to CnI allows for minimally invasive delivery of cells within a three-dimensional construct into the irregularly-shaped injury cavity.

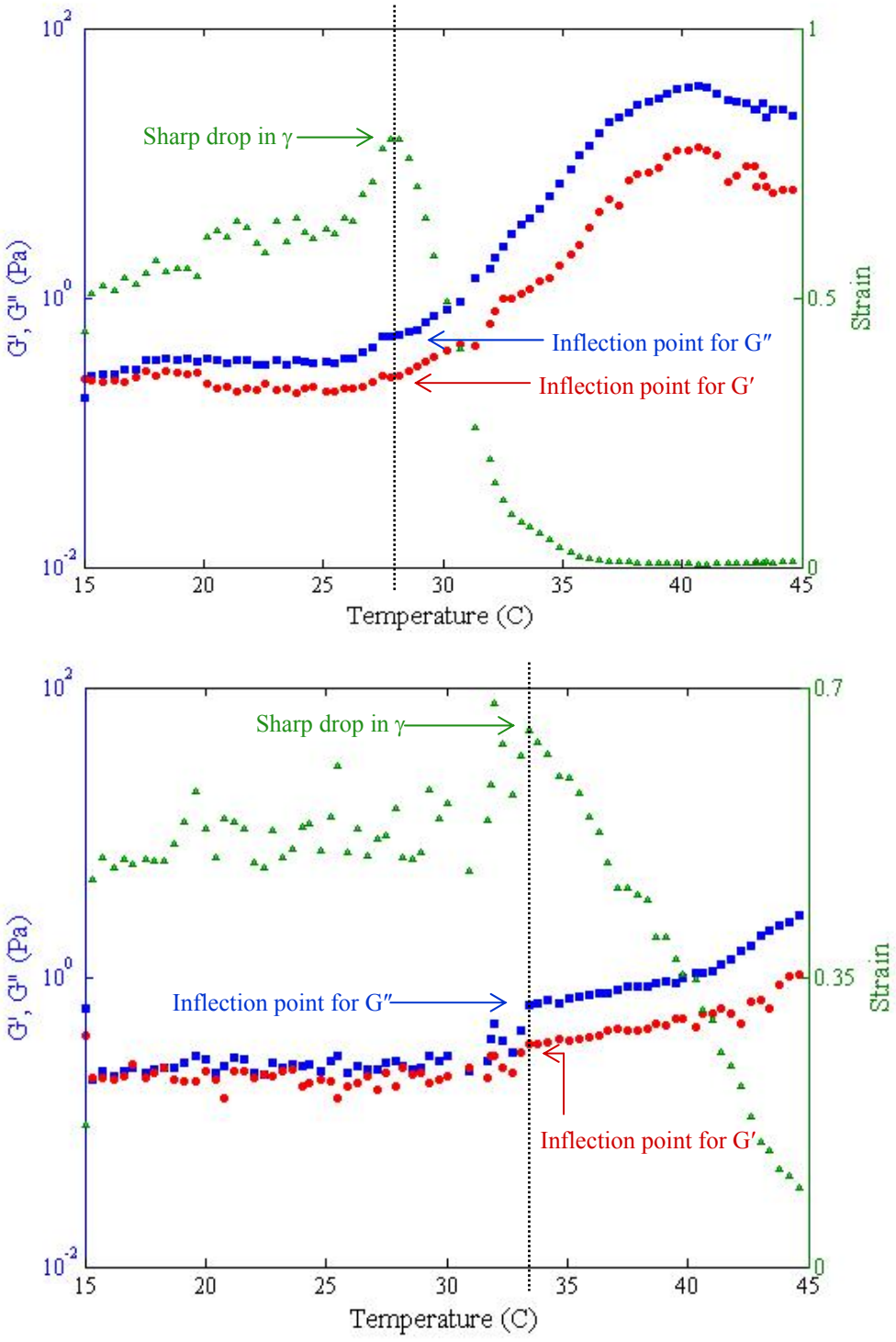

Figure 2.2: Gelation curve of scaffolds used to deliver NSC

Plot of storage (G′) and loss (G″) moduli and strain (γ) versus temperature, showing that the gelation of the FN/CnI mixture (top) occurs at 27.8±1.0ºC, and the gelation of the

LN/CnI mixture (bottom) occurs at 34.1±0.9 ºC. Thus, both scaffolds transition from a viscous liquid to a soft gel upon reaching body temperature.

Mice treated with tissue engineered construct containing laminin have improved cognitive function

Injured mice performed significantly worse than uninjured sham animals throughout the MWM task which began at 5 weeks post-transplant ($p<0.005$). By the end of the testing period (days 11-12), mice that were treated with NSC+LN/CnI showed significant improvement compared to untreated animals ($p=0.03$). An intermediate treatment effect was observed for mice that were treated with NSC only or NSC+FN/CnI, as these were not significantly different from either injured or sham groups. Furthermore, the recovery for the group treated with NSC+LN/CnI is due to the combination of the NSC with the LN-based construct, as the LN/CnI only treatment was significantly worse than both sham ($p=0.01$) and NSC+LN/CnI ($p=0.001$) groups by the end of the testing period. These data are summarized in the plot in **Figure 2.3**.

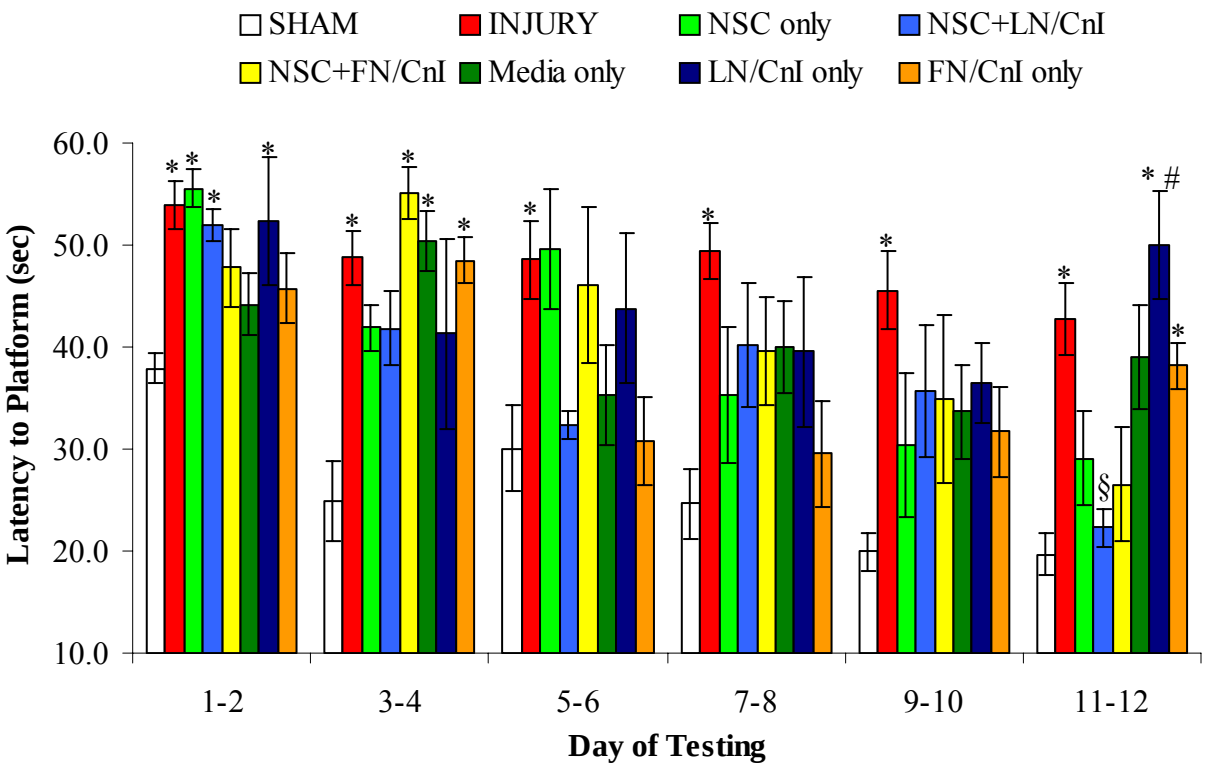

Figure 2.3: Morris water maze

Morris water maze testing was conducted at 5 weeks post-transplant. By the end of the testing period (days 11-12), injured animals were significantly worse than uninjured shams. While all the treatment groups containing cells seemed to improve performance on this spatial learning task, only the mice treated with NSC in the LN-based scaffold were significantly better than untreated animals. *$p<0.01$ vs. SHAM, §$p=0.03$ vs. INJURY, #$p=0.001$ vs. NSC+Ln/CnI; Mean ± SEM

Cells delivered in tissue engineered constructs have enhanced survival

Cell counts of surviving GFP^+ cells (**Figure 2.4**) revealed that at 1 week post-transplant only 2-3% of the donor cells survived, independent of initial delivery conditions. These low survival rates are consistent with other transplant work in the injured or diseased brain (Barker et al., 1996; Lindvall, 2003; Saporta et al., 1999). By 3 weeks post-transplant, there was a slight drop in surviving cells for all the groups (again no difference among treatments). However, by 8 weeks post-transplant, cells delivered within the FN-based scaffold showed improvement in survival (1,530±220 GFP^+ cells) compared to NSC transplants in media alone (550±440 GFP^+ cells, $p=0.06$), and cells delivered within the LN-based scaffold (2,520±690 GFP^+ cells) showed significantly improved survival compared to those transplanted in media alone ($p=0.005$). Thus, the delivery conditions used in this study did not protect cells from the initial drop in survival, but did help maintain the survival of cells remaining after the acute loss.

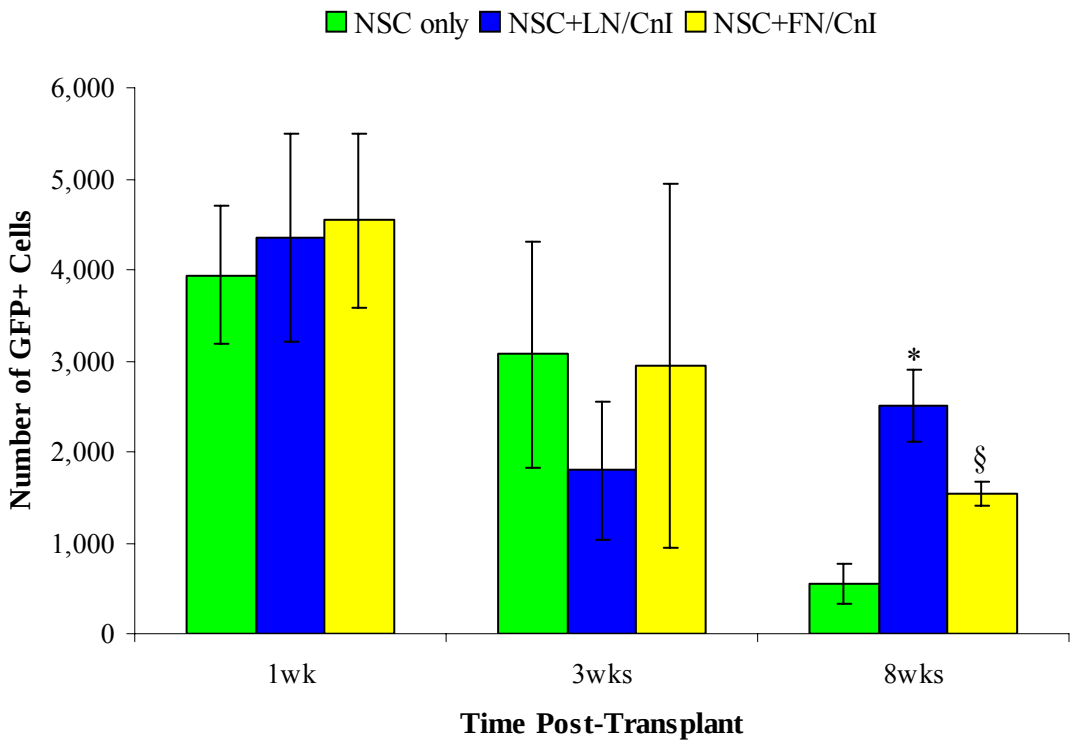

Figure 2.4: Survival of GFP$^+$ donor cells

By 8 weeks post-transplant, NSC seeded in the FN-based scaffold had better survival than NSC transplanted alone and NSC seeded in the LN-based scaffold seemed to have survival than cells delivered within the FN-based scaffold and had significantly better survival than NSC transplanted alone. *p=0.005 (NSC only vs. NSC+LN/CnI), §p=0.06 (NSC only vs. NSC+FN/CnI); Mean ± SEM

Phenotype, proliferation, and migration are consistent among treatment groups

The phenotype of the transplanted cells was investigated by probing for the following markers: nestin and Sox-1 (neural progenitor cells), GFAP (reactive astrocytes), β-tubulin III (immature neurons), NeuN (nucleic marker of neurons), NG2 and PDGF receptor-α (oligodendrocyte progenitor cells), O4 (immature oligodendrocytes), CNPase (oligodendrocytes). Of the brain sections assessed, no GFP^+ cells were found to double label for nestin, Sox-1, β-tubulin III, NeuN, PDGF receptor-α, O4, or CNPase. Regardless of treatment conditions or time post-transplant, a small subset (3-7%) of donor cells were found to be either $GFAP^+$ or $NG2^+$, indicating a fraction of the NSC differentiated into glial phenotypes (data not shown). As far as donor cell proliferation, none of the GFP^+ cells were found to be positive for the mitosis marker H3, indicating cells were likely not undergoing proliferation at 1, 3, or 8 weeks post-transplant (data not shown). This does not rule out the possibility that proliferation occurred at other time points. However, given the cell survival data, it is not likely that the NSC were proliferating after transplantation.

Analyzing the profiles of the GFP^+ cells with respect anatomical location and degree of dispersion, no differences in migration were observed between treatment groups (**Figure 2.5**). At 1 and 3 weeks post-transplant, GFP^+ cells were found either bordering the cavity or in the tissue immediately adjacent to the cavity. By 8 weeks, cells were found distributed within the hippocampus tissue for all treatment groups, indicating surviving cells migrated to this slightly deeper structure. One notable difference was that GFP^+ cells delivered within the FN/CnI scaffold were more clustered together at 1 and 3 weeks post-transplant than cells delivered in media or the LN/CnI scaffold. This is likely due to the fact that the FN/CnI scaffold forms a gel at 27.8±1.0ºC, which is well below physiologic temperature. However, the LN/CnI scaffold gels near physiologic

temperature, thus may not have completely gelled immediately after injection, allowing cells to align the cavity as they do when transplanted within media.

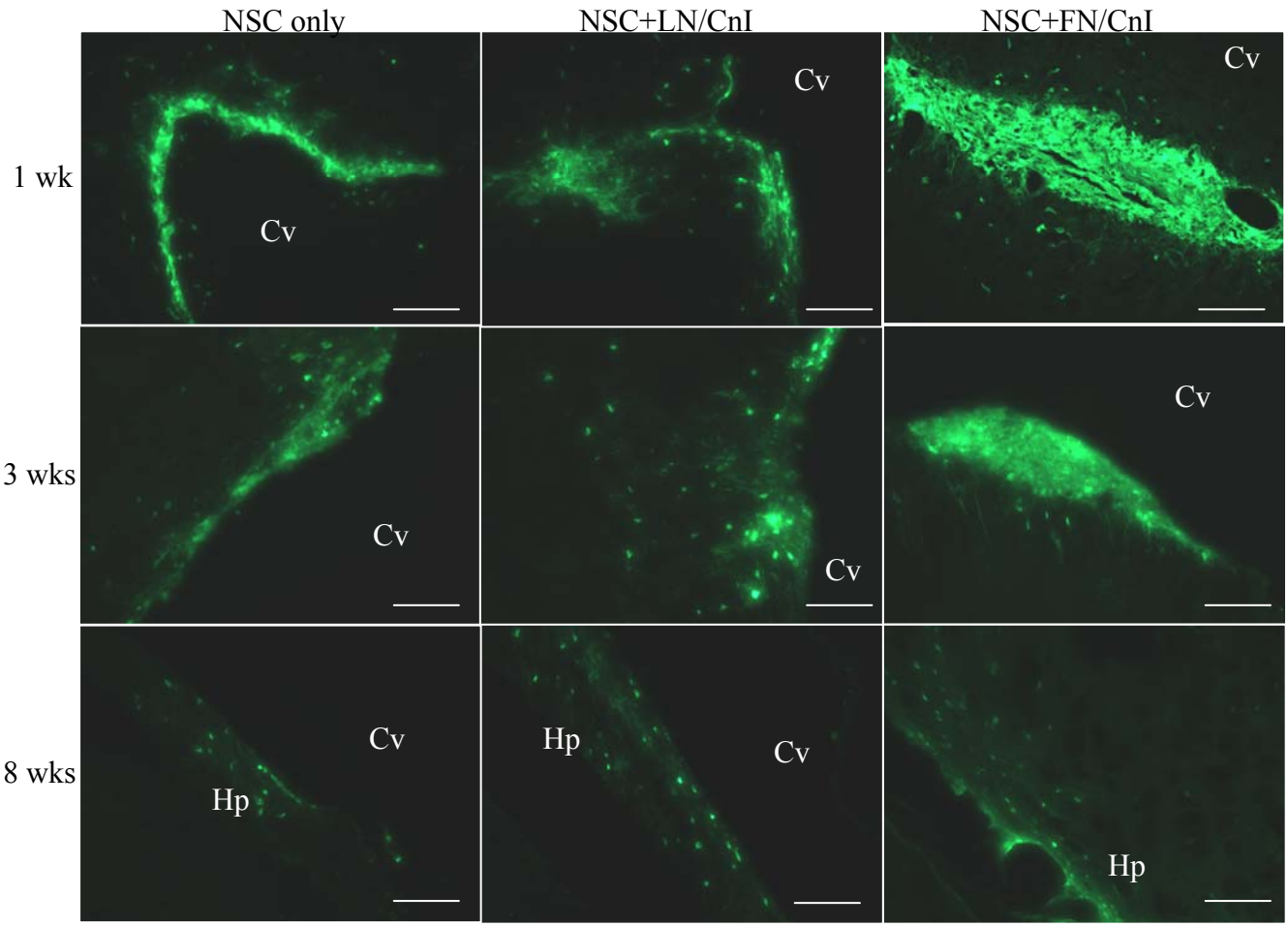

Figure 2.5: Migration profile of GFP$^+$ donor cells

Representative micrographs are shown of the GFP+ cells in the three treatment conditions at 1, 3 and 8 weeks post-transplant. At 1 and 3 weeks, cells are found clustered (or in a large clump in the case of delivery with FN/CnI) either lining the injury cavity (Cv) or in the tissue immediately adjacent. By 8 weeks, remaining GFP+ cells are found distributed within the hippocampus (Hp). No differences in anatomical location or radial migration of the cells were observed between the different treatment groups. Scale bar = 100μm

Discussion

We have demonstrated that delivering NSC within specific ECM protein-based scaffolds improves donor cell survival and behavioral recovery. When NSC were transplanted into the injury cavity at 7 days post-injury, the initial donor cell survival was poor (2-3% cells surviving by 1 week post-transplant), regardless of transplant conditions. It is possible that for all treatment groups, the early drop in cell numbers was caused by the initial delivery conditions (i.e. shear stress from passing through the needle), the cytotoxic environment of the injured tissue, or an immune response to the donor cells. The fact that this lack of initial survival is observed for multiple cell transplantation paradigms necessitates further investigation into the mechanisms governing initial cell death. Interestingly, the use of an appropriate ECM protein based scaffold seems to affect the cell death rate of the donor cells that do survive the initial stress of the delivery. Our previous work showed that transplanting NSC within a FN/CnI-based scaffold had improved survival by 3 weeks post-transplant, and in this study, NSC transplanted within a FN/CnI or LN/CnI scaffold had improved survival by 8 weeks.

One of the attractive features of cell transplantation therapy is the ability to provide a treatment with a single, minimally-invasive procedure, that can be sustained in the brain tissue and evolve with the dynamic environment. Thus, enhancing later phase survival is important to maintain the presence of these donor cells throughout the recovery period. Since the scaffolds used in this study are likely not present by 8 (or even 3) weeks post-transplant, it is remarkable that the acute transplant conditions have such a profound effect on non-acute graft survival. There are a number of potential explanations for this later phase difference in cell survival, relating to how the scaffold affects initial donor cell behavior. Delivering NSC has the potential to provide a heterogeneous population of cells to the injured brain, but it is likely that the majority of the cells are a common phenotype since they are exposed to similar extracellular cues. It is possible that the

microenvironment of the ECM-based scaffold selects for more robust cell types. In addition, since both FN and LN are involved in neural development and are found in increased levels following brain injury, these proteins may be affecting the host cells, subsequently creating a more favorable environment for surviving donor cells.

Incorporating the thermally reversible protein CnI into our tissue engineered construct allowed for minimally-invasive delivery of cells, while providing a three-dimensional support scaffold in situ. This system enables the construct to conform to an irregularly-shaped injury cavity and still provide physical support to donor cells. We chose to incorporate CnI into the scaffold based on the gelation properties of this ECM protein; which provided a structural for the FN or LN and subsequently the NSC. Because we were focusing on improving the behavior of the transplanted NSC, and CnI did not enhance adhesion or migration of these cells in vitro, we focused on the FN or LN as the bioactive scaffold components. However the contribution of CnI cannot be ignored. Collagen I hydrogels have been used in vitro to support the proliferation and differentiation of neural stem and progenitor cells (Ma, 2004). Thus, the CnI transplanted with the NSC may also provide initial support for the donor cells.

The similarities in migration patterns between treatment groups was unexpected since previous studies transplanting NSC within a similar FN/CnI scaffold found enhanced migration by 3 weeks post-transplant. However, the cells from that study were used at passage 2 (versus 3), thus may have been more migratory. In both studies, cells migrate to the hippocampus. It is possible that the presence of the ECM scaffold selects for more migratory cells that move to the hippocampus at earlier time points than cells transplanted alone, and this occurred between 3 and 8 weeks in the study presented here. Notably, we have previously shown that performance on the Morris water maze spatial

learning task is correlated with the number of surviving donor cells in the hippocampus (Shear et al., 2004); and this was corroborated by the work presented here.

The future use of LN versus FN in transplant paradigms seems more appealing since cells delivered in the LN/CnI scaffold had increased survival compared to those delivered in the FN/CnI scaffold. Furthermore, only the group treated with the construct containing both NSC and LN was significantly better than the untreated group in a spatial learning task. Further exploration utilizing LN-based scaffolds may continue to improve graft survival and integration, and ultimately promote functional recovery. Our laboratory is currently examining cross-linking LN or LN peptides to a thermo-reversible polymer (methylcellulose) for use as a scaffold (Stabenfeldt et al., 2006). This system allows us to better control the microenvironment of the donor cells, and will enable advancements such as increasing the density of functional LN peptides, altering the scaffold degradation rate, or incorporating neurotrophic growth factors in order to appropriately support and direct NSC.

In summary, we have utilized tissue engineering strategies to improve survival of grafted cells, which resulted in cognitive recovery. Inspired by the success of fetal tissue transplants, we engineered a construct containing NSC supported physically and chemically by ECM proteins. This design can be exploited for a number of CNS disorders which could also benefit from increased survival of transplanted NSC, including spinal cord injury and stroke. Tissue engineering allows for enhanced control over transplanted cells, and will likely prove useful in augmenting cell transplantation therapy in the CNS.

Acknowledgements

Assistance with the rheometer was provided by Sarah Stabenfeldt.

CHAPTER 3

FIBRONECTIN AND LAMININ INCREASE IN THE MOUSE BRAIN FOLLOWING CONTROLLED CORTICAL IMPACT INJURY

Abstract

The complex environment of the traumatically injured brain exhibits aspects of inhibition and ongoing cell death together with attempts at repair and regeneration. Elucidating these events and exploiting factors involved in endogenous repair and regeneration may aid in developing more effective treatments for traumatic brain injury. Two extracellular matrix proteins critical to neural development - fibronectin and laminin - may also play a protective or reparative role in the injury response. While both of these proteins have been found to increase following human brain injury, the presence of these proteins has not been studied in a clinically-relevant animal model of blunt head trauma. In this study, we examined the spatiotemporal profile of both fibronectin and laminin in the mouse brain following controlled cortical impact injury. Fibronectin and laminin reactivity was localized to the injury penumbra up to 14 days post-injury and was significantly higher than uninjured controls at 3 days post-injury. Upon examining the spatial relationship of fibronectin and laminin to support cells, we found macrophages/activated microglia prominently present in the fibronectin-rich tissue, consistent with a role for fibronectin in facilitating debris clearing. Furthermore, reactive astrocyte processes were found sheathing laminin positive vasculature, suggesting that laminin may play a role in repairing the blood-brain barrier. These and other hypothesized reparative roles for fibronectin and laminin after traumatic brain injury are discussed.

Introduction

Following traumatic brain injury (TBI) there is a dynamic interplay among factors promoting repair and regeneration in competition with factors inhibiting such functions. It is important to understand mechanisms across this continuum to develop treatments that enhance cell survival and regeneration. Many different extracellular matrix (ECM) proteins have been found to increase following brain injury. While much attention has been devoted to identifying ECM proteins that inhibit regeneration, particularly chondroitin sulphate proteoglycans (for review, see Properzi et al., 2003), there are ECM proteins in the injured brain that may be beneficial to injured and regenerating tissue. Two ECM proteins of particular interest are fibronectin (FN) and laminin (LN). Human studies have shown an increase in both FN (Liu and Sturner, 1988) and LN (Hausmann and Betz, 2000) following TBI. An increase in FN has also been seen in various experimental brain insults, including penetrating stab wound (Egan and Vijayan, 1991; Mizutani and Kimura, 1987), cortical cold injury (Nag et al., 1997; Suzuki and Choi, 1990), and kainic acid lesion (Hertel et al., 2000), while LN has been found in increased levels after cortical cold injury (Kakinuma et al., 1998; Suzuki and Choi, 1990) and penetrating stab wound (Szabo and Kalman, 2004). However, neither of these proteins has been evaluated using a model of blunt trauma, the most common type of head injury in humans (Langlios, 2004). Because of the physical and chemical complexities associated with blunt trauma, it is important to evaluate the early injury response in appropriate animal models. Two well-studied models of TBI in rodents are the fluid percussion and controlled cortical impact (CCI) injury, both of which mimic many pathological aspects of human closed head injury, such as cell death in multiple locations in the brain and prolonged cognitive and motor deficits (Dixon et al., 1991; Fox et al., 1998). In this study, we examined the spatiotemporal profile of both FN and LN following moderate CCI injury in mice.

Methods

Controlled Cortical Impact Injury

Adult male C57BL6 mice (6-10 weeks, Jackson Laboratory, Bar Harbor, MN) were anesthetized with an intraperitoneal injection of ketamine (62 mg/kg) and xylazine (50 mg/kg) and placed in a stereotaxic frame. After exposing the skull, a 4 mm craniectomy was performed over the left fronto-parietal cortex, with the center at 1.0 mm posterior to bregma and 1.5 mm lateral to the midline. CCI was produced with a 3 mm pneumatically-operated metal impactor which enters the brain at a velocity of 6.0 m/sec. The impact to the brain occurred at a depth of 1.0 mm below the dura matter layer and the impactor remained in the brain for 150 msec. The impactor rod was angled 15° to the vertical to be perpendicular to the tangential plane of the brain curvature at the impact surface. A linear variable displacement transducer connected to the impactor recorded velocity and duration to verify the consistency of the injuries. After the CCI injury, the incision was sutured and animals were placed on warm pads for recovery. Sham injury surgeries consisted of anesthesia injection, skull exposure, craniectomy, and suturing. All mice were housed in a temperature-controlled environment with a 12:12-hr light-dark cycle with food and water available ad libitum. All procedures conformed to guidelines set forth in the Guide for the Care and Use of Laboratory Animals and were approved by the Georgia Institute of Technology Institutional Animal Care and Use Committee.

Histology

Mice were sacrificed for analysis at the following post-injury time points: 1 day (n=5), 3 days (n=5), 7 days (n=4), 14 days (n=2), and 7 weeks (n=2). In addition, sham (uninjured; n=3) brains that underwent all surgical procedures excluding the impact were also immunostained to establish baseline levels of ECM expression. Mice were re-anesthetized with an intraperitoneal injection of ketamine (124 mg/kg) and xylazine (100 mg/kg), then perfused with 0.1M phosphate buffered saline (PBS), followed by 4%

paraformaldehyde. The brains were harvested, immersed overnight in 4% paraformaldehyde at 4°C, then cryoprotected with 30% sucrose. The brains were then embedded in optimal cutting temperature (OCT) medium (Sakura, Torrence, CA), frozen, and stored at -80°C. A cryostat (Richard-Allan Scientific, Kalamazoo, MI) was utilized to serially section the brain into 16 μm thick cut coronal sections.

Immunohistochemistry was performed to detect FN, LN and glial fibrillary acidic protein (GFAP; a marker of reactive astrocytes). Briefly, sections were rinsed in PBS, and then blotted and permeabilized in 8% goat serum and 0.1% tritonX-100 in PBS for 1 hr at room temperature. Sections were then incubated overnight at 4°C with the following antibodies (Chemicon, Temecula, CA) for FN (AB2033, 1:50), LN (AB2034, 1:100) and/or GFAP (MAB360, 1:400). Following rinsing in PBS, sections were incubated in the corresponding fluorescent conjugated secondary antibodies (Sigma-Aldrich, St. Louis, MO, and Jackson ImmunoResearch, West Grove, PA) for 2 hours at room temperature. To identify macrophages and reactive microglia, fluorescent-conjugated isolectin IB_4 was used for its ability to bind carbohydrates on these phagocytic cells, as previously described (Maddox et al., 1982; Pennell et al., 1994). The conjugated isolectin IB_4 (1:100; Molecular Probes, Eugene, OR) was added just as the primary antibody described above. Note that for double staining, antibodies were applied simultaneously. Fluorescent microscopy was used to visualize staining.

Quantification and Statistics

The areas of FN^+ and LN^+ tissue were quantified separately using Image Pro® software (Media Cybernetics, Silver Spring, MD). Briefly, the total area of positively stained tissue above a threshold intensity (set by the highest intensity for that brain's immunohistochemical negative controls) was determined for each section. The total area of ECM^+ tissue per section was then averaged for a given brain and then for all the brains

from each time point. These areas were compared to the amount of positive staining in uninjured control brains using a general linear model ANOVA (Minitab, State College, PA), followed by Tukey's pairwise comparisons. An alpha value of 0.05 was used to assess if means were significantly different.

Results

Spatiotemporal Profile of Fibronectin and Laminin

Fluorescent microscopy revealed that both FN and LN were consistently present in brain tissue adjacent to the injury site up to 14 days post-CCI injury, and there was negligible immunolabeling for FN or LN in other areas of the brain, consistent with previous findings that normal adult brain tissue contains low levels of these ECM proteins (Bellail et al., 2004; Maenpaa et al., 1997). In the injury penumbra, the FN^+ tissue was distributed throughout the extracellular space in a seemingly random arrangement (**Figure 3.1–A**). LN^+ tissue was along vasculature within the injured cortex adjacent to the injury site (**Figure 3.1–B**). Quantification of the amount of positively stained tissue revealed that the amount of both positive tissue was significantly higher than uninjured sham brains at 3 days post-CCI injury for both FN ($p=0.02$) and LN ($p=0.03$) (**Figure 3.2**).

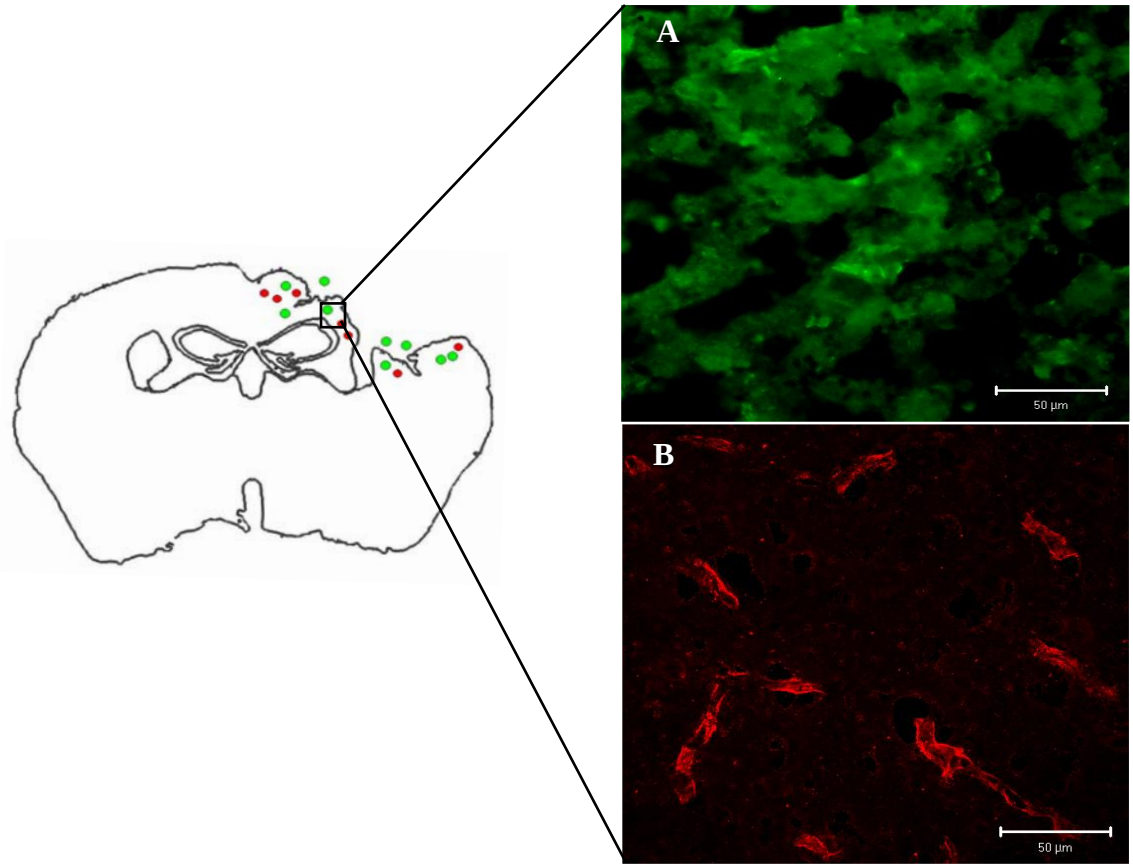

Figure 3.1: Spatial localization of FN and LN post-injury

Both FN and LN are distributed around the tissue adjacent to the cavity. Morphology of FN^+ tissue (A, green) appeared to be distributed in the extracellular space between neural cells, and LN^+ tissue (B, red) appears to surround vasculature. Images are at 3 days post-CCI injury; Scale bar=50µm

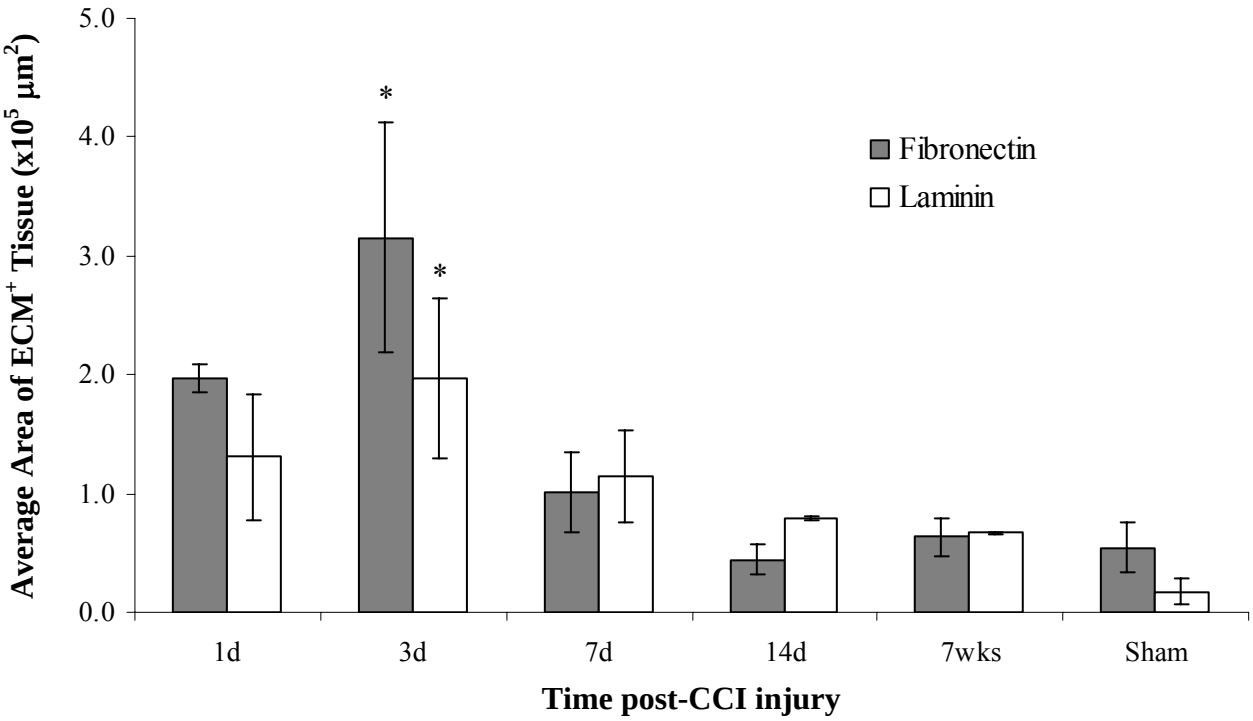

Figure 3.2: Temporal profile of FN^+ and LN^+ tissue in the injury penumbra following CCI injury

Immunoreactivity of FN and LN was significantly higher than uninjured controls at 3 days following CCI injury ($p<0.05$). Mean ± SEM

Spatial Proximity of Fibronectin and Laminin to Support Cells

To determine if support cells were interacting with these ECM proteins in the injured tissue, we double labeled for FN and LN with isolectin IB_4 to identify macrophages or activated microglia and with GFAP antibody to identify reactive astrocytes. Isolectin+ cells exhibiting a rounded morphology (indicative of either macrophages or activated microglia) were found bordering the lesion at 3 days and in greater numbers at 7 days post-CCI injury, which is a similar pattern to that seen in contused human brains (Holmin et al., 1998). At 3 and 7 days post-CCI injury, the majority of isolectin IB_4^+ cells could be seen in areas of FN-rich tissue adjacent to the site of injury (**Figure 3.3-A**). As expected, $GFAP^+$ cells were seen bordering the injury site at all time points examined, and the intensity and density were maximal at 7 days post-CCI injury. Up to 7 days post-CCI injury, many of these $GFAP^+$ cells were found bordering the LN stained areas (**Figure 3.3-B**). Specifically, the LN^+ tissue coincided with microvasculature near the injury site, and astrocytic processes appeared to surround the circumference of these LN^+ vessels (arrows in **Figure 3.3-B**).

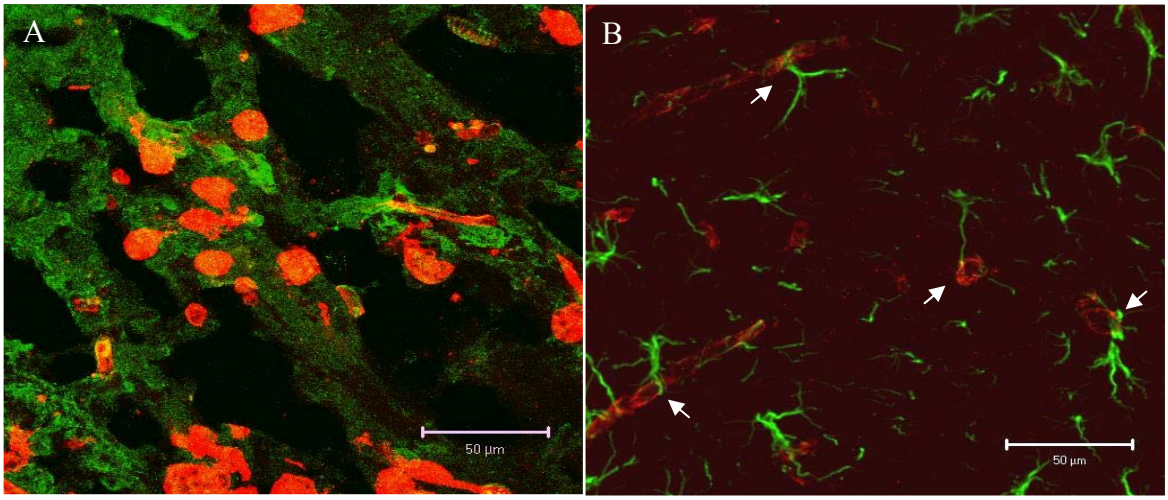

Figure 3.3: Co-spatialization of FN and LN with support cells

A. The majority of macrophages/activated microglia (red) were found in regions of FN^+ tissue (green); B. Activated astrocytes (green) are often found in proximity to LN^+ tissue (red). Note that reactive astrocytes wrap the LN^+ vasculature (arrows in B). Images taken at 3 days post-CCI injury; Scale bars = 50µm

Discussion

We evaluated the spatiotemporal profile of two ECM proteins, FN and LN, following CCI injury. This was the first analysis of these proteins using a model of blunt TBI. We found both proteins increase in the injury penumbra up to 1 week post-injury, and the area of ECM^+ tissue was significantly higher than uninjured brains at 3 days post injury. In addition, we determined that these proteins correlate spatially with support cells. Specifically, FN correlates with macrophages or activated microglia and LN correlates with reactive astrocytes.

This spatial proximity of FN and isolectin+ phagocytic cells may result because both monocyte-derived macrophages and at least some FN are blood borne, and permeate into the injured brain tissue during acute increases in blood-brain barrier (BBB) permeability (Giulian et al., 1989; Nag et al., 1997). Moreover, this could point to a functional relationship similar to that seen in cutaneous wound healing, where FN acts as an opsonin for phagocytes, thereby aiding in the clearance of dead tissue and debris (Martin et al., 1988), and accelerating the healing process (Lariviere et al., 2003). Further investigation is warranted to determine if this spatial proximity is strictly due to their common source or if there is a functional, and moreover, beneficial relationship.

The correlation of $GFAP^+$ reactive astrocytes with LN^+ vessels indicates a role for LN in restoring vasculature and the BBB. Laminin increases also correlate temporally with the biphasic opening of the BBB which occurs within the first 6 hours and again at 3 days post-injury (Baskaya et al., 1997). Perhaps astrocytes are secreting LN (Freire et al., 2004) to temporarily seal breaches in the BBB. An alternative explanation is that the LN^+ vasculature corresponds with the reformation of basal laminae during angiogenesis (Davis and Senger, 2005; Grant et al., 1990) and the close proximity of reactive

astrocytes is due to restoration of the BBB formed by astrocytic end feet (Risau and Wolburg, 1990).

In addition to the aforementioned hypothesized functions based on the spatial overlap of FN with macrophages/activated microglia and LN with reactive astrocytes, there are other plausible mechanisms for these ECM proteins in the injured brain, including regenerative and pro-survival roles. For example, both FN and LN have been shown to promote cell migration, neurite extension and synapse formation in the developing brain (Bi et al., 2001; Einheber et al., 1996; Tsuru et al., 1996). Since the injured brain recapitulates many aspects of development (e.g., neuro- and gliogenesis, angiogenesis, increase in ECM proteins), FN and LN may be aiding in regeneration. For example FN in the extracellular tissue may act as a substrate for the migration of astrocytes and microglia to the site of injury. Since FN and LN are supportive for neurite outgrowth, their presence may be an endogenous attempt to compete with inhibitory components of found in the extracellular environment. Or, FN and LN may aid in synapse formation as neighboring neurons compensate for the damaged / lost networks. However, by the time regeneration or plasticity are favorable (after the initial volatile spread of injury), the expression of these proteins returns to normal.

Another potential reparative mechanism for FN in the injured tissue is decreasing apoptosis. Fibronectin has been shown to be anti-apoptotic for many cell types in vitro (Fukai et al., 1998; Zhang et al., 1995), including neurons (Gibson et al., 2005); and plasma FN null mice had increased apoptosis following in vivo ischemia (Sakai et al., 2001). It has been found in vitro that FN binding to $\alpha_5\beta_1$ integrin increases expression of Bcl-2, which subsequently decreases apoptosis (Fukai et al., 1998; Gibson et al., 2005; Zhang et al., 1995). Since the $\alpha_5\beta_1$ integrin is expressed by adult neural cells (King et al.,

2001; Pinkstaff et al., 1999), FN may aid in salvaging cells in the injury penumbra via anti-apoptotic signaling.

In further support of a beneficial role for FN and LN in the injured brain is the therapeutic use of these proteins in experimental brain injury. Functional peptides from both FN (Yanaka et al., 1996) and LN (Yanaka et al., 1997) have been shown to reduce polymorphic leukocyte accumulation and infarct size and improve neurological outcome following cerebral ischemia. In addition, LN-based hydrogels encouraged cell infiltration and angiogenesis and reduced glial scar formation when implanted into a brain cavity (Hou et al., 2005).

In summary, this study examined the spatiotemporal profile of FN and LN in the injured brain using a clinically relevant model of blunt trauma. We found that both proteins increase in the injury penumbra at acute time points and are spatially associated with support cells. Based on these associations, as well as the roles of FN and LN in neural development and other peripheral systems, it is hypothesized that these proteins have reparative roles, which are summarized in **Figure 3.4** (FN) and **Figure 3.5** (LN). This hypothesis needs to be further examined to better understand how these ECM proteins fit into the complex, interdependent pathology. It is important to determine the mechanisms of action underlying repair/regeneration as well as inhibition since functional treatments for TBI will likely target a combination of actions.

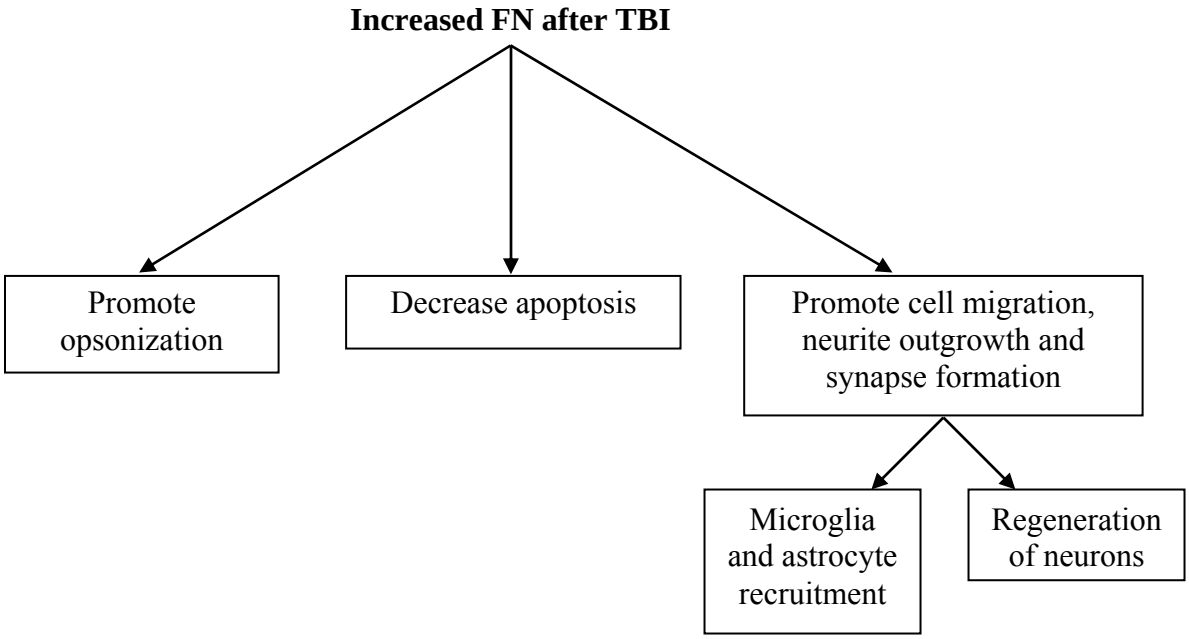

Figure 3.4: Potential mechanisms for fibronectin in the injured brain

This is an overview of possible reparative mechanisms for FN in response to traumatic brain injury. Based on the co-spatialization of FN with macrophages / activated microglia and the role of FN in wound healing and tissue repair in the periphery, FN may be aiding in phagocytosis of injured tissue. Based on the information that FN is anti-apoptotic for various cell types, including neurons, and the fact that adult neural cells express integrins for FN indicate a potential role in reducing apoptosis of cells vulnerable to secondary cell death. Finally, based on the critical role of FN in neural development, and the observations that the brain attempts to re-develop following injury, FN may be promoting migration, neurite outgrowth and synapse formation for regenerating neurons, or may be recruiting glial cells, such as microglia and astrocytes, to the site of injury.

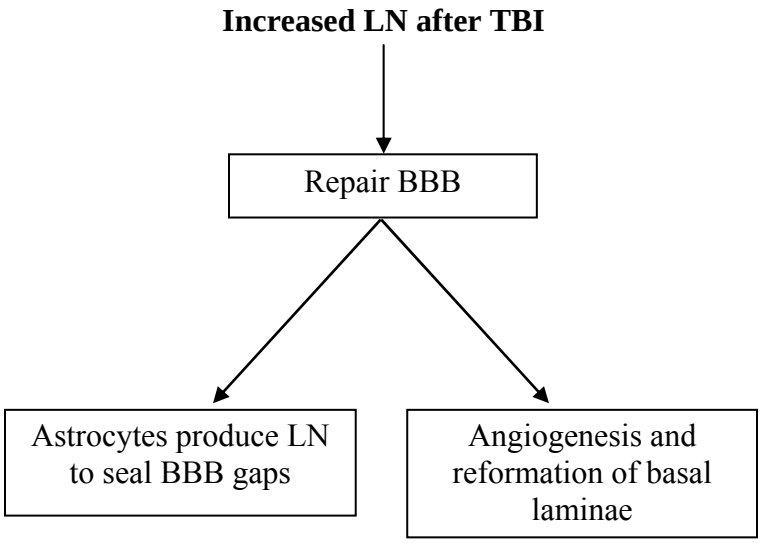

Figure 3.5: Potential mechanisms for laminin in the injured brain

This is an overview of possible reparative mechanisms for LN in response to traumatic brain injury. Based on the fact that LN was found in increased amounts around vasculature and reactive astrocytes were found wrapping the circumference of these LN^+ vessels a role for LN in restoring the blood-brain barrier is hypothesized. Astrocytes may secrete LN to temporarily seal gaps in the blood-brain barrier and/or LN may be involved in promoting angiogenesis and the reformation of the tight junctions.

CHAPTER 4

PLASMA FIBRONECTIN IS NEUROPROTECTIVE FOLLOWING TRAUMATIC BRAIN INJURY

Abstract

Certain extracellular matrix components inhibit neural regeneration in the traumatically injured brain. In the case of the matrix protein fibronectin, traumatic brain injury leads to increased levels of plasma-derived fibronectin in the brain tissue. Using conditional plasma fibronectin (pFN) knockout mice, we determined that pFN is neuroprotective following traumatic brain injury. Injured mice deficient in pFN performed significantly worse on motor and cognitive tasks, had significantly increased lesion volume and apoptotic cell death, and had significantly less phagocytic cells in the injured cortex compared to injured mice with normal pFN. Moreover, intravenous injections of fibronectin prior to the injury rescued the neural deficits seen in the pFN deficient mice. These results demonstrate that fibronectin is neuroprotective to the injured brain and identify a novel target for therapeutic interventions.

Introduction

Traumatic brain injury (TBI) remains a major health and socioeconomic problem in the United States (Langlios, 2004). Developing effective treatment strategies has been difficult due to the complex microenvironment of the traumatically injured brain, where processes that promote repair and regeneration (e.g., neurogenesis, angiogenesis) compete with mechanisms of secondary damage and inhibition (e.g., excitotoxicity, increase in chondroitin sulphate proteoglycans (CSPGs)). Significant attention has been given to the extracellular matrix (ECM) environment in the injured brain, primarily due to inhibitory properties of certain ECM proteins (e.g., CSPGs, Nogo) (Properzi et al.,

2003). However, other components of the ECM environment, such as fibronectin (FN) may aid in repair and support regeneration. Fibronectin promotes cell migration, neurite outgrowth and synapse formation during neural development (Reichardt and Tomaselli, 1991), but is largely absent in adult brain tissue (Maenpaa et al., 1997). However, levels of FN increase following both clinical (Liu and Sturner, 1988) and experimental (Egan and Vijayan, 1991; Hertel et al., 2000; Nag et al., 1997) TBI. These FN increases in the traumatically injured brain are likely involved in repair since FN plays a reparative role in wound healing (Grinnell, 1984), has been shown to be anti-apoptotic for many cells in vitro, including neurons (Gibson et al., 2005), and is beneficial following brain ischemia (Sakai et al., 2001).

Fibronectin is encoded by a single gene, and alternative splicing generates different isoforms. Two major forms of FN are found in the body: cellular FN, which is produced in a variety of cell types and is immobilized in ECM, and plasma FN (pFN) which is made by hepatocytes and secreted in soluble form into the blood (Kornblihtt et al., 1985). Since the elevated fibronectin levels after TBI are largely due to an increase in blood-brain barrier (BBB) permeability and cellular FN is not expressed in wounded tissue following ischemic brain injury (Sakai et al., 2001), it is hypothesized that the majority of FN found after TBI is plasma borne. Thus, the use of conditional adult pFN null mice allows us to investigate for the first time the role of FN in the traumatically injured brain. Plasma FN null mice were compared to wild type (WT) littermates following a moderate controlled cortical impact (CCI) injury. Furthermore, a subset of the pFN null mice received intravenous (IV) injections of pFN prior to the injury to determine if differences were due specifically to the absence of pFN. Animals were compared on the basis of motor and cognitive behavior, cell death profiles, and response of phagocytic cells.

Methods

Plasma Fibronectin Knockout Mice

Plasma FN null mice (129Sv/C57BL6; breeder pairs generously provided by H.P. Erickson and R.O. Hynes) were generated via the *Cre-loxP* conditional gene knockout system as previously described (Sakai et al., 2001). Briefly, mice containing a floxed (fl; *loxP*-site containing) *FN* allele were crossed with mice expressing *Cre* under the control of the interferon and polyinosidic-polycytidic acid (pIpC) *Mx* promoter. This generates mice carrying the null allele (*FN*(fl/fl)/*Mx-Cre*$^{+}$; *FN*(fl/fl) mice carrying the *Mx-Cre* transgene). This system has been shown to completely delete the *FN* gene in the liver (Sakai et al., 2001), where pFN production occurs. The deletion of the *FN* gene was induced in male mice at least 8 weeks old by 3 intraperitoneal injections of 250 μg pIpC at 2 day intervals. Mice were used for experimentation 1 month after the last pIpC injection. Blood samples were taken from all animals to verify the absence of pFN for knockout-induced mice using western blot analysis. Note that *FN*(fl/fl)/*Mx-Cre*$^{+}$ with pIpC are referred to as "pFN null" and *FN*(fl/fl)/*Mx-Cre*$^{-}$ littermates with pIpC are considered "WT" for these studies. All mice were housed in a temperature-controlled environment with a 12:12-hr light-dark cycle with food and water available ad libitum. All procedures conformed to guidelines set forth in the Guide for the Care and Use of Laboratory Animals and were approved by the Georgia Institute of Technology Institutional Animal Care and Use Committee.

Genotyping

All animals used for this study (including breeders) were genotyped to identify the presence or absence of the *Mx-Cre* transgene. Tissue samples for genetic analysis were obtained from the tails of 3-5 week old mice. DNA was extracted from the tissue using a DNeasy® Tissue Kit (Qiagen, Valencia, CA) and quantified using the PicoGreen® dsDNA Quantification Kit (Invitrogen, Carlsbad, CA). DNA was amplified by

polymerase chain reaction (PCR) using the primers for Cre forward (5'-ACTATCCAGCAACATTTGGGCCAG-3') and Cre reverse (5'-GATCCTGGCAATTTCGGCTATACG-3'); primers for Beta5 forward (5'-CTACAGCATACCTGCTTCACTGTG-3') and Beta5 reverse (5'-GCCTTTCTGTGGGTTCCA-3') to use as internal controls (all primers from Invitrogen). Amplified DNA samples were run through electrophoresis using a 2% agarose gel (Fisher, Hampton, NH) and labeled with ethidium bromide (Kodak, New Haven, CT) for visualization under ultraviolet light (see sample in **Figure 4.1**)

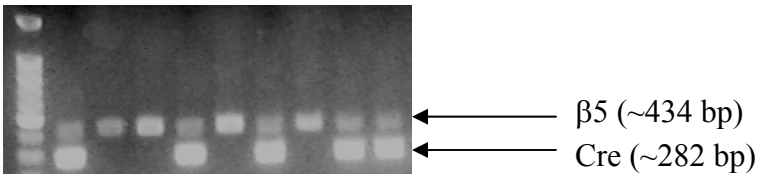

Figure 4.1: Agarose gel for electrophoresis of DNA

Heterozygous FN(fl/fl)/*Mx*-*Cre*$^{+/-}$ mice were used for this study, thus DNA samples from all animals were genotyped to identify the presence of the *Cre* transgene (as shown above). All study mice received injections of polyinosidic-polycytidic acid, which promotes the transcription of *Cre* on the *Mx* promoter, and thus the deletion of *FN*. Complete deletion of the *FN* gene in the liver occurs in *Cre*$^{+}$ mice ("pFN null"), and no deletion of the FN gene occurs in *Cre*$^{-}$ mice ("wild type").

Plasma Fibronectin Administration

Soluble human pFN (Invitrogen) was injected into the bloodstream for a subset of the pFN null mice to test if changes in injured pFN null mice are reversed by replacing FN, For rodents, physiological concentrations of pFN are 580μg/ml (George et al., 1993) and the half-life ranges from 15-30 hours (Gauperaa and Seljelid, 1985; Kiener et al., 1986). Thus, to approximate "normal" pFN circulation in the blood, an IV injection (via the tail vein) of soluble pFN (~50mg/kg) was given to the mice 6 hours prior to surgery. Blood samples were collected at the time of injury to determine the amount of pFN in circulation using western blot analysis. Plasma FN null mice that received IV injections of pFN are referred to as "pFN null + pFN IV".

Western Blot Analysis

Blood drawn via the saphenous vein (~50 μl) was spun for 10 minutes at 2,300 cgf to separate the plasma from the cellular components, for subsequent western blot analysis. Proteins from denatured plasma samples (50 μg of total protein based on micro bicinchoninic acid protein analysis) were separated with standard SDS-PAGE, followed by protein transfer onto nitrocellulose membranes. Membranes were incubated in blocking buffer (5% nonfat dry milk, 0.1% Tween20, and 0.02% NaN_3 in phosphate buffered saline (PBS)) overnight at 4°C. Membranes were then blotted with FN antibody (F3648, 1:1000; Sigma-Aldrich, St. Louis, MO) for 1 hour at room temperature, followed by rinsing and blotting with an alkaline phosphatase-conjugated secondary antibody (1:10,000; Jackson ImmunoResearch, West Grove, PA) for 1 hour at room temperature. After the addition of ECF substrate (Amersham, Buckinghamshire, UK), bands were detected using a fluorescent image analyzer (Fuji FLA-3000, Stamford, CT). Band intensities were quantified (Adobe Photoshop, Adobe Systems, San Jose, CA) and averaged for a each group on a given blot in order to assess the relative amounts of pFN in the pFN null and pFN null + pFN IV groups compared to WT animals.

Controlled Cortical Impact Injury

Adult male mice (14-28 weeks old) were anesthetized with 3% isoflurane and secured in a stereotaxic frame, where anesthesia was maintained (via gas mask) with 1-2% isoflurane. After exposing the skull, a 4 mm craniectomy was performed over the left frontoparietal cortex (center at -2.0 mm AP and +2.0 mm ML to bregma). A pneumatically-operated metal impactor (diameter = 3 mm) impacted the brain at a velocity of 6.0 m/sec reaching a depth of 1.0 mm below the dura matter layer and remained in the brain for 150 msec. The impactor rod was angled 15° to the vertical to be perpendicular to the tangential plane of the brain curvature at the impact surface. A linear variable displacement transducer (Macrosensors, Pennsauken, NJ) connected to the impactor measured velocity and duration to verify consistency of the injuries. After the CCI, the incision was sutured when all bleeding had ceased. Sham injury surgeries (for uninjured controls) consisted of anesthesia, scalp incision, craniectomy, and suturing.

Motor Behavior

The rotarod and beam walk tasks were used as measures of motor ability. For both tasks, mice were given three trials per time point, and the mean value was used in the data analysis. Mice were allowed to rest for at least 30 seconds between trials for both training and testing. Testing occurred at 1 day pre-CCI injury (baseline), at 3 and 7 days post-CCI injury, then weekly up to 10 weeks post-CCI injury, except during the period of MWM testing. Note that sham injury groups included uninjured WT (n=11), pFN null (n=4), and pFN null + pFN IV mice (n=4). For analysis purposes, these groups were combined into one sham group because there was no significant difference between them ($p>0.05$).

Note that previous studies have indicated that uninjured 129SvEMS mice perform significantly worse on the beam walk task compared to uninjured C57BL6 mice (Fox et al., 1999). Thus, a preliminary study was performed to assess the motor ability of the mice used in this study which are on a C57BL6x129Sv back-strain by comparing uninjured C57BL6x129Sv (*FN*(fl/fl), *Mx/Cre*$^{-}$) mice (n=15) with uninjured C57BL6 mice (n=9; Jackson Laboratory, Bar Harbor, MN) on both the rotarod (10 and 20 rpm) and beam walk (6 mm) tasks. In addition, to control for effects of pIpC administration, uninjured C57BL6x129Sv (*FN*(fl/fl), *Mx/Cre*$^{-}$) mice that received pIpC injections (n=13) were also compared. Results of this preliminary study are shown in **Figure 4.2**, revealing no significant differences between the different strains of mice on the 10 rpm rotarod task ($p>0.9$) or the 6 mm beam walk task ($p>0.2$). However, the uninjured C57BL6x129Sv (*FN*(fl/fl), *Mx/Cre*$^{-}$) mice were significantly better than uninjured C57BL6 mice on the 20 rpm rotarod task ($p<0.001$). As for the effects of the pIpC drug administration used in this study, there were no differences between mice that received pIpC injections compared to the same strain of mice that did not receive the pIpC injections for both of the rotarod tasks and the beam walk task.

Rotarod

A rotarod apparatus with automatic fall detection (Columbus Instruments, Columbus, OH) was used to assess gross motor function, specifically hind limb control and grip strength (Fox et al., 1998). All mice were trained over four consecutive days prior to baseline assessments. During training, mice were placed on the rod rotating at either 5 or 10 rpm and removed from the rod at 60 seconds if they have not fallen prior to this time. Training includes four trials per day at 10 rpm, and an additional two trials at 5 rpm speed prior to beginning the 10 rpm trials on the first day (to acclimate the mice to the rotarod). For testing, mice were placed on a spindle rotating at constant speed of 20 rpm (for 3 trials).

Latency to fall (height = 25 cm) was recorded, and if the animal did not fall within 60 seconds, it was removed from the rod.

Beam Walk

A narrow balance beam was employed to measure fine motor coordination (Fox et al., 1998). Mice were trained 1 day prior to surgery, and testing for baseline levels began after all animals had been trained. The animals were placed at a start point near the suspended end of a narrow beam (width of first 50 cm = 12 mm, width of second 50 cm = 6 mm, height from padded surface = 25 cm) and allowed to traverse the beam 100 cm into a darkened goal box. Since injury to the left frontoparietal cortex is involved in motor control for the right hindlimb, the outcome measure was the percentage of the total right hindlimb steps that are foot faults (when the paw slips 5 mm or more below the beam surface).

Cognitive Behavior – Morris Water Maze

A MWM task was used to assess spatial learning performance at week 6 post-CCI injury (Morris, 1984). The MWM apparatus consisted of a white, circular tank (diameter = 1 m) filled with opaque water (20±1°C; nontoxic white tempura paint) to a depth of 52 cm (23 cm from the top of the tank). A platform was submerged to a depth of 1 cm and placed 30 cm from the wall of the pool in the center of the northeast quadrant. The position of the platform remained constant throughout the study. Each animal was given 2 trials per day with a 30 minute inter-trial interval for 12 consecutive days. At the start of each trial, the mouse was placed in the pool (facing the pool wall) at one of four randomly determined starting positions ("N, S, E, W"). Over the course of 4 trials (2 days) all positions were tested, thus data are reported as the average latency to platform over these 4 trials. Each mouse was allowed to swim freely in the pool until it found the hidden platform or until 60 seconds had elapsed, and a video tracking system (San Diego

Instruments, San Diego, CA) was utilized to measure the latency to the platform. If a mouse did not find the platform in 60 seconds, it was manually guided to the platform, where mice were allowed to rest for 15 seconds before being removed from the pool. A probe-trial using a visible platform to assess visuomotor abilities was conducted following the last MWM trial, and mice that did not find the visible platform in less than 55 seconds were removed from the analysis. As stated above, sham injury groups from WT (n=6), pFN null (n=3), and pFN null + pFN IV mice (n=4) were combined for analysis purposes because there was no significant difference between them ($p>0.05$).

Note that previous studies have indicated that 129SvEMS do not learn to find the hidden platform in the MWM maze task (Fox et al., 1999). Thus, a preliminary study was performed to assess the spatial learning ability of the mice used in this study, which are on a C57BL6x129Sv back-strain, by comparing uninjured C57BL6x129Sv (*FN*(fl/fl), *Mx/Cre*$^{-}$) mice (n=15) with uninjured C57BL6 mice (n=9) on the MWM. In addition, to control for effects of pIpC administration, uninjured C57BL6x129Sv (*FN*(fl/fl), *Mx/Cre*$^{-}$) mice that received pIpC injections (n=13) were also compared. Results of this preliminary study are shown in **Figure 4.2**, indicating that all groups tested significantly decrease their latency to find the hidden platform by testing days 5-6 ($p<0.05$ versus days 1-2), and there are no differences between the groups tested at any testing day ($p>0.2$). Thus, we can conclude there was not a significant contribution of either the back-strain or the pIpC drug administration used in this study to spatial learning ability on MWM task.

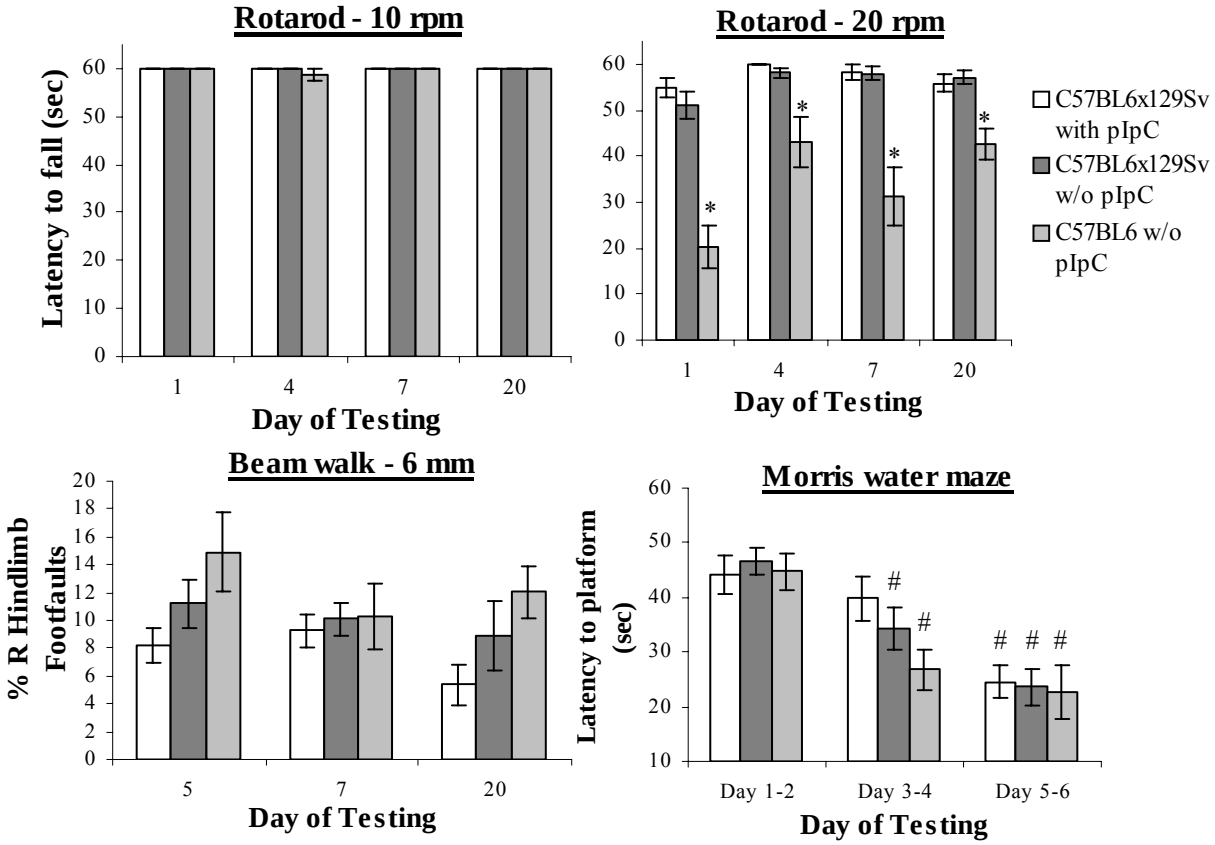

Figure 4.2: Behavioral comparison of C57BL6x129Sv mice with and without pIpC and C57BL6 mice

Preliminary behavior studies revealed no effect of giving the pIpC drug to the C57BL6x129Sv (*FN*(fl/fl), *Mx/Cre*$^{-}$) mice for any of the behavior tasks examined. Also, there were no differences between uninjured C57BL6x129Sv (*FN*(fl/fl), *Mx/Cre*$^{-}$) mice (with or without pIpC) and uninjured C57BL6 mice on the 10 rpm rotarod, the 6 mm beam walk task, or the MWM task. However, on the 20 rpm rotarod task, the uninjured C57BL6x129Sv (*FN*(fl/fl), *Mx/Cre*$^{-}$) mice both with and without pIpC were significantly better than uninjured C57BL6 mice (*$p<0.001$). Mean ± SEM; #$p<0.05$ versus testing days 1-2.

Tissue Harvesting and Preparation

All animals were re-anesthetized with an intraperitoneal injection of pentobarbital (80 mg/kg). Mice were perfused with 0.1M PBS (pH=7.4) followed by 4% paraformaldehyde fixative. Brains were post-fixed with 4% paraformaldehyde for approximately 12 hours, and cryoprotected in phosphate-buffered 30% sucrose. The brains were then embedded in optimal cutting temperature (OCT) medium (Sakura, Torrence, CA), frozen, and stored at -80°C. Using a Microtome Cryostat (Richard-Allan Scientific, Kalamazoo, MI) the brains were sliced into 25 μm coronal sections and mounted onto sequential series gelatin-coated glass slides for histological analysis.

Immunohistochemistry for Fibronectin in Brain

Sections (spaced 100 μm apart) were stained for FN to assess how the absence of pFN correlated with the amount of FN in the injured brain. Briefly, sections were rinsed in PBS, and then blotted and permeabilized in 8% goat serum and 0.1% tritonX-100 in PBS for 1 hr at room temperature. Sections were then incubated overnight at 4°C with an antibody recognizing mouse FN (AB2033, 1:50, Chemicon, Temecula, CA). Following rinsing in PBS, sections were incubated in a rhodamine-conjugated secondary antibody (Jackson ImmunoResearch) for 2 hours at room temperature. The area of immunostained tissue was quantified for 1-2 representative brains at 3 and 7 days post-CCI using Image Pro® software (Media Cybernetics, Silver Spring, MD). Briefly, the total area of positively stained tissue above a threshold intensity (set by the highest intensity for that brain's immunohistochemical negative controls) was determined for each section. The total area of FN^+ tissue per section was then averaged for a given brain

Lesion Volume Assessment

Tissue sections were stained for Nissl with cresyl violet (Sigma-Aldrich, St. Louis, MO). The Cavalieri volume estimator (Rosen and Harry, 1990) with Stereo Investigator

software (Microbrightfield, Williston, VT) was used to determine the volume of the injury cavity (20-24 sections total at 200 μm increments per brain; coefficient of error ≤ 0.1).

Identification of Apoptotic Cell Death and Phagocytic Cell Response

Though apoptosis can persist long after the initial insult, the majority occurs within the first few days (Newcomb et al., 1999). To assess apoptotic cell death at 1 and 3 days post-CCI injury, tissue sections were stained with TUNEL using the NeuroTACS™ II kit (Trevigen, Gaithersburg, MD). Because we have previously found that phagocytic cells appear at the injury site at 3 and 7 days post-CCI injury, we probed for phagocytic cells at these time points. Phagocytic cells were labeled with fluorescently tagged isolectin IB_4, which binds to the terminal α-D-galactosyl residues on microglia and macrophages (Maddox et al., 1982; Pennell et al., 1994). Under microscopic analysis cells that were both $TUNEL^+$ and exhibited morphological characteristics of apoptosis (e.g., condensed, rounded cytoplasm and apoptotic bodies) were considered apoptotic; and cells that were both isolectin IB_4^+ and demonstrated a rounded, amoeboid shape were counted as phagocytic cells. We did not distinguish between monocyte derived (blood-borne) macrophages and activated microglia.

Quantification of Apoptotic and Phagocytic Cells

Design-based stereology was used to quantify both the number of apoptotic cells in the injured cortex (defined as the area of cortical tissue within 1 mm from the cavity border) and the number of macrophages/activated microglia in both injured cortex and blood clots (which were often present at the injury site at 3 days post-injury). The optical fractionator method (Gundersen, 1986; Sterio, 1984) with Stereo Investigator software (Microbrightfield) was employed to obtain these estimates (20-28 sections at 200 μm increments per brain; coefficient of error ≤ 0.1).

In Vitro Assessment of Fibronectin and Survival of Astrocytes

Astrocytes were harvested from cortices of rat pups (post natal day 0-1), and allowed to mature in vitro. Cultures were maintained in Dulbecco's modified eagle medium: nutrient mixture F-12 (DMEM/F12, 1:1, Invitrogen) supplemented with 10% goat serum (Invitrogen), which was replenished every 2-3 days, and cells were passaged when they reached 80-90% confluency (approximately every 5 days). When cells were passaged for the tenth time, they were plated in plastic wells coated with either poly-l-lysine (PLL; 50 μg/ml, n=4; Sigma-Aldrich) or FN (10 μg/ml, n=4; Invitrogen). When cells reached confluency (after 2 days) media was replaced with DMEM/F12 only (without serum), which has been shown to induce apoptotic cell death for multiple cell types (Baehrecke, 2005; Gibson, 1999; Kulkarni and McCulloch, 1994). After 24 hours in serum-free media, cells were rinsed in PBS, fixed with 4% paraformaldehyde, and stained with calcein-AM to identify live cells and ethidium homodimer to identify dead or dying cells (Invitrogen). Micrographs were taken under fluorescent microscopy of 5 regions within each well: center, "northwest", "northeast", "southwest", and "southeast", which were subsequently quantified to determine the percentage of dead/dying cells.

Statistics

A general linear model ANOVA, followed by Tukey's pairwise comparisons (Minitab, State College, PA) were used to compare the following groups: 1- sham (for behavior studies only), 2- injured WT, 3- injured pFN null, and 4- injured pFN null + pFN IV, or for the in vitro portion, 1- astrocytes plated on PLL and 2- astrocytes plated on FN. An alpha value of 0.05 was used to assess if the means were significantly different. Data are reported as mean ± SD.

Results

Plasma fibronectin null mice lack fibronectin in blood and injured brain

Because FN is crucial to organ formation in embryonic development, a conventional FN knockout animal is embryonically lethal (George et al., 1993). Thus, the *Cre-loxP* system was employed to accomplish the deletion of *FN* in adult animals (Sakai et al., 2001). The *FN* gene in the liver of mice expressing *Cre* driven by an interferon- or pIpC-inducible *Mx* promoter was completely deleted upon receiving pIpC, thus eliminating the source of pFN (Sakai et al., 2001). Western blots of plasma samples taken at 3 and 7 days and 1 month following the final injection of pIpC confirm that the levels of FN in the pFN null mice are less than 2% of that found in WT mice (**Figure 4.3**). To further confirm that any effects of the *FN* knockout on the brain injury response were due specifically to the absence of pFN, a subset of the pFN null mice received a single IV injection of pFN (~50mg/kg) 6 hours prior to the CCI injury. Western blots of plasma samples taken shortly after the injury revealed that the level of FN in these mice was 55±12% of the amount measured in WT mice (**Figure 4.3**).

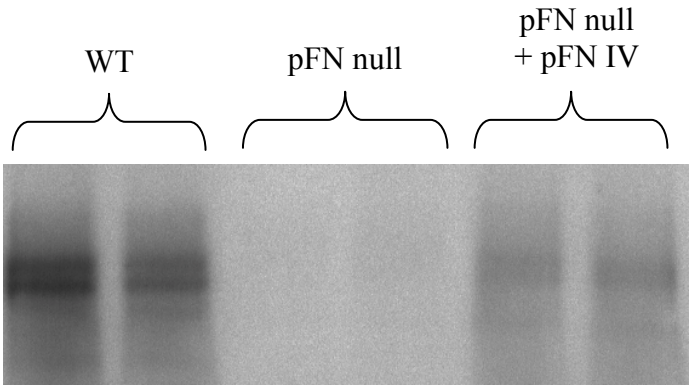

Figure 4.3: Fibronectin in blood plasma

The amount of FN in the blood plasma in adult pFN null mice is less than 2% of the amount found in wild type littermates by 3 days after initiation of the knockout. Injecting human pFN intravenously 6 hours prior to CCI injury restores the amount of FN in the plasma to 55±12% of the amount in found in wild type animals.

Following TBI, FN enters the brain during acute breaches in the BBB (Nag et al., 1997), thus the amount of FN in the bloodstream is proportional to the amount of FN in the injured brain tissue. We have previously found that there is an increased amount of FN in the injury penumbra up to 7 days following CCI injury in mice (Tate et al., Submitted 2006). Immunolabeling for FN in the injured brain of pFN null mice at 1, 3 and 7 days post-CCI injury revealed that there are negligible levels of FN in the injured brain tissue of pFN null mice, which was confirmed by quantification at 3 and 7 days post-CCI injury (**Figure 4.4**). This suggests that the majority of the FN in the injured brain is plasma borne. While there is incomplete deletion of the FN gene in the brain (Sakai et al., 2001) and increases in FN gene expression have been observed at 3 days post-CCI injury (Natale et al., 2003), any contribution of cellular FN produced by neural cells, such as astrocytes, or macrophages was undetectable by the immunohistochemistry techniques employed.

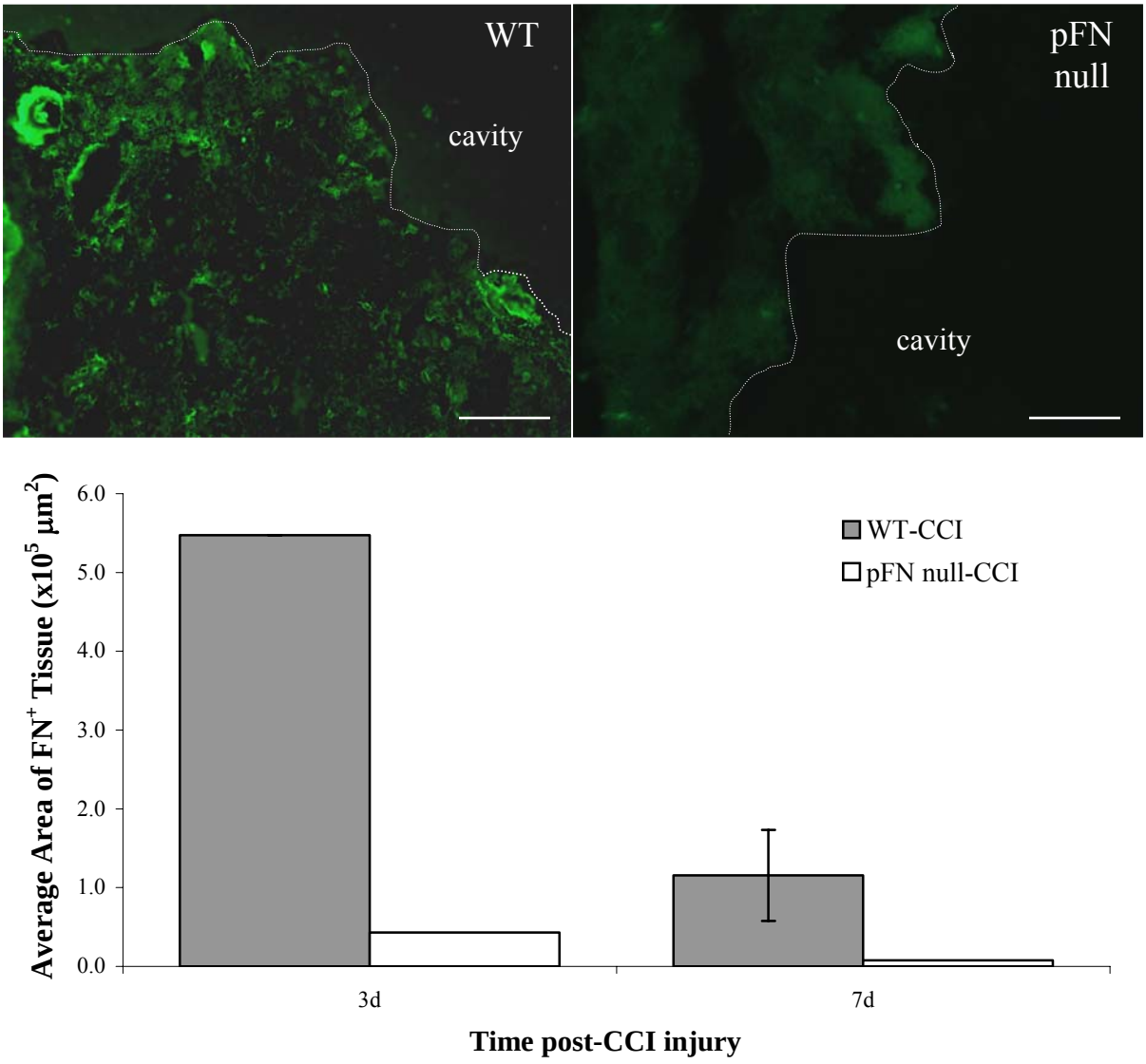

Figure 4.4: Fibronectin in the injured brain

Previous work showed that fibronectin is increased in the injured brain at acute time points following controlled cortical impact injury. Immunolabeling for fibronectin revealed that the protein was found in injury penumbra for wild type animals (top, left), but not for pFN null mouse brains (top, right). Cavity border is indicated by dotted line. Images taken at 3 days post-CCI injury; Scale bars = 100 µm; Quantification of 1-2 representative brains at 3 and 7 days post-CCI injury is shown in the bottom plot, confirming that there is not a noteworthy amount of fibronectin positive tissue in injured brains of pFN null mice. Mean ± SEM

Plasma fibronectin null mice exhibit worse motor and cognitive performance following brain injury

Motor behavior was assessed weekly (up to 10 weeks post-CCI injury) using the rotarod and beam walk tasks. For the rotarod task, an injury deficit was only observed up to 7 days post-CCI injury, and there were no differences between injured groups in gross motor ability (data not shown). However, on the beam walk task the moderate impact to the frontoparietal cortex resulted in an injury deficit throughout the testing period for all injured groups compared to sham animals ($n=19$, $p<0.05$). Interestingly, the injured pFN null group ($n=10$) performed significantly worse than both the injured WT ($n=11$, $p<0.05$) and pFN null + pFN IV ($n=10$, $p<0.05$) groups up to 4 weeks post-CCI injury on the 12 mm beam (**Figure 4.5**-top), and up to 9 weeks post-CCI injury on the more difficult 6 mm beam (**Figure 4.5**-bottom). Moderate cortical injuries result in impaired spatial learning ability, likely due to secondary cell death and damage in the hippocampus. Therefore, animals were tested on the Morris water maze (MWM) task at 6 weeks post-CCI injury to assess spatial learning ability (12 day, 2 trials per day testing paradigm). By testing days 9-10, the injured pFN null group ($n=5$) performed significantly worse than both the injured WT ($n=5$, $p<0.01$) and pFN null + pFN IV ($n=3$, $p<0.01$) groups, and were also significantly worse than the injured pFN null + pFN IV group ($p=0.027$) at testing days 7-8 (**Figure 4.6**). While an injury deficit compared to sham animals ($n=13$) was observed for the injured WT ($p<0.05$) and pFN null groups ($p<0.05$), there was no statistical difference between the sham and injured pFN null + pFN IV groups. Overall, the increased motor and cognitive deficits for the pFN null mice that were reversed by replacing pFN clearly indicate a protective role for FN in TBI.

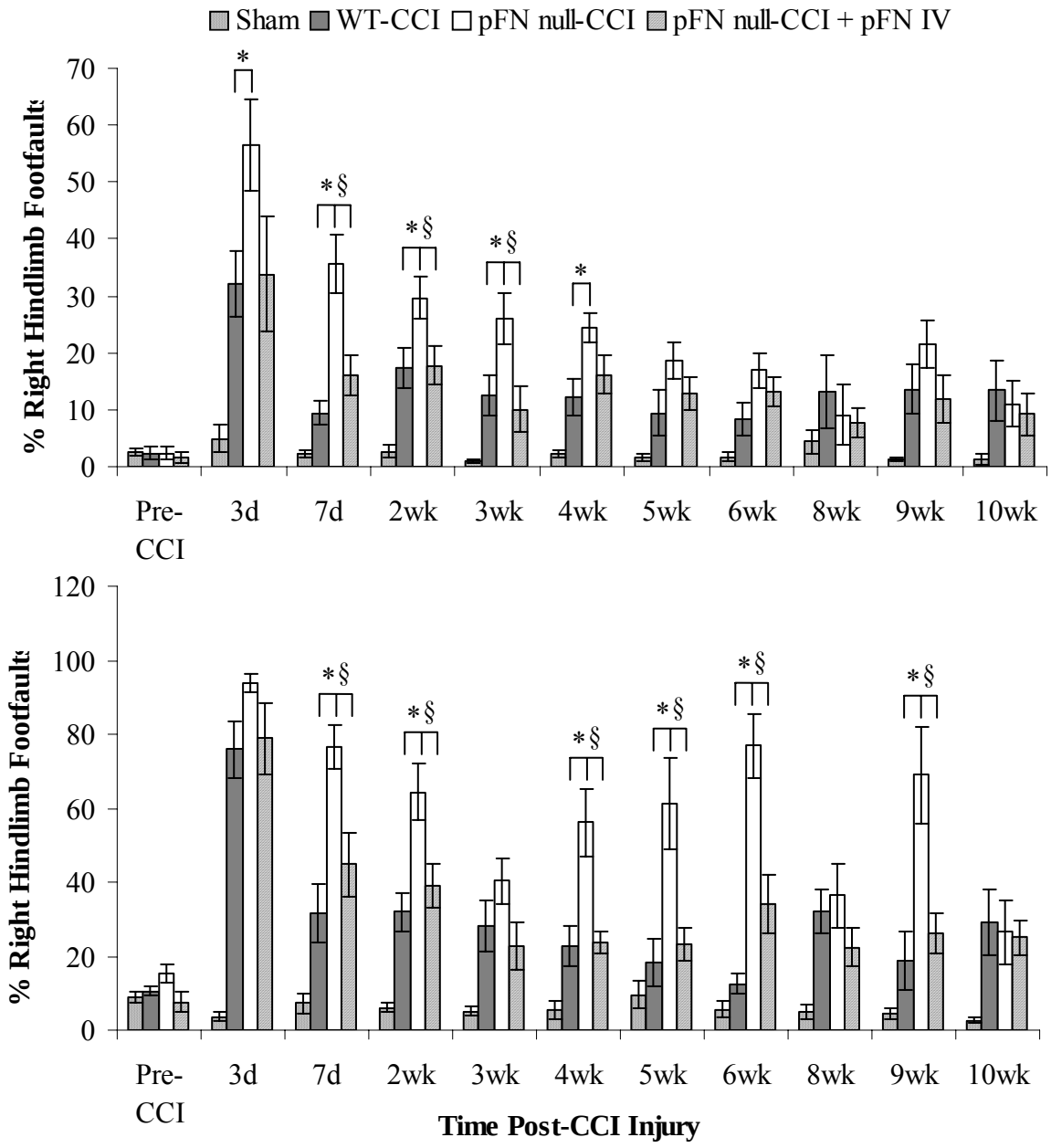

Figure 4.5: Beam walk

Injured mice deficient in plasma FN perform significantly worse compared to both injured WT mice and pFN null mice receiving an IV pFN injection on the 12 mm (top) and 6 mm (bottom) beam walk task. *p<0.05 pFN null-CCI vs. WT-CCI; §p<0.05 pFN null-CCI vs. pFN null-CCI + pFN IV; Mean ± SEM

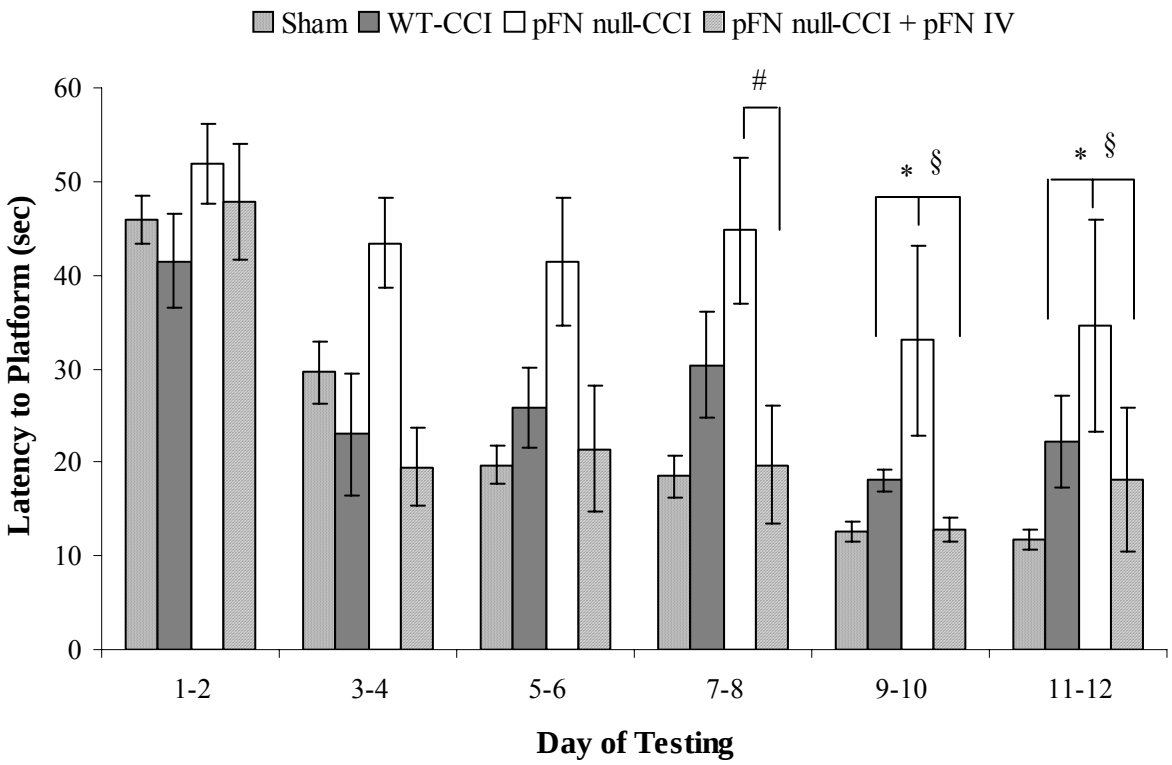

Figure 4.6: Morris water maze

At 6 weeks post-CCI, injured mice deficient in pFN perform significantly worse at the end of the testing period compared to both injured WT mice and injured pFN null mice receiving an IV pFN injection. Mean ± SEM; *$p<0.01$ pFN null-CCI vs. WT-CCI; $^{\S}p<0.01$ pFN null-CCI vs. pFN null-CCI + pFN IV, $^{\#}p=0.03$ pFN null-CCI vs. pFN null-CCI + pFN IV

Plasma fibronectin null mice have increased cell death following brain injury

Lesion volume was quantified at 10 weeks post-CCI injury using Nissl staining and the Cavalieri estimator. The lesion volume for the injured pFN null mice (8.7±1.9 mm^3, n=4) was significantly higher than both the injured WT (4.2±0.6 mm^3, n=4, p=0.003) and pFN null + pFN IV (4.0±0.8 mm^3, n=3, p=0.004) groups (**Figure 4.7**). Therefore, mice that only lack pFN (and thus FN in the injured brain) had lesions over two times as large as normal mice by 10 weeks post-injury, which is on the order a million of more cells lost in the pFN null brain. Since the mechanism of tissue loss is important in understanding the response to injury, apoptotic cells in the ipsilateral cortex were identified at 1 and 3 days post-CCI injury using terminal deoxynucleotidyl-transferase-mediated biotin-dUTP nick end labeling (TUNEL) and morphological criteria to distinguish apoptosis from necrosis. Quantification of apoptotic cells, obtained using unbiased stereology, revealed that the injured pFN null mice had increased cortical cell death. At 3 days-post CCI, the number of apoptotic cells in the injured cortex of pFN null mice (29,700±11,720, n=5) was 5-fold higher than for injured WT mice (5,800±4,910, n=6, p=0.002 and almost 3-fold higher than for injured pFN null mice with pFN IV (11,150±6,370, n=3, p=0.028) (**Figure 4.8**).

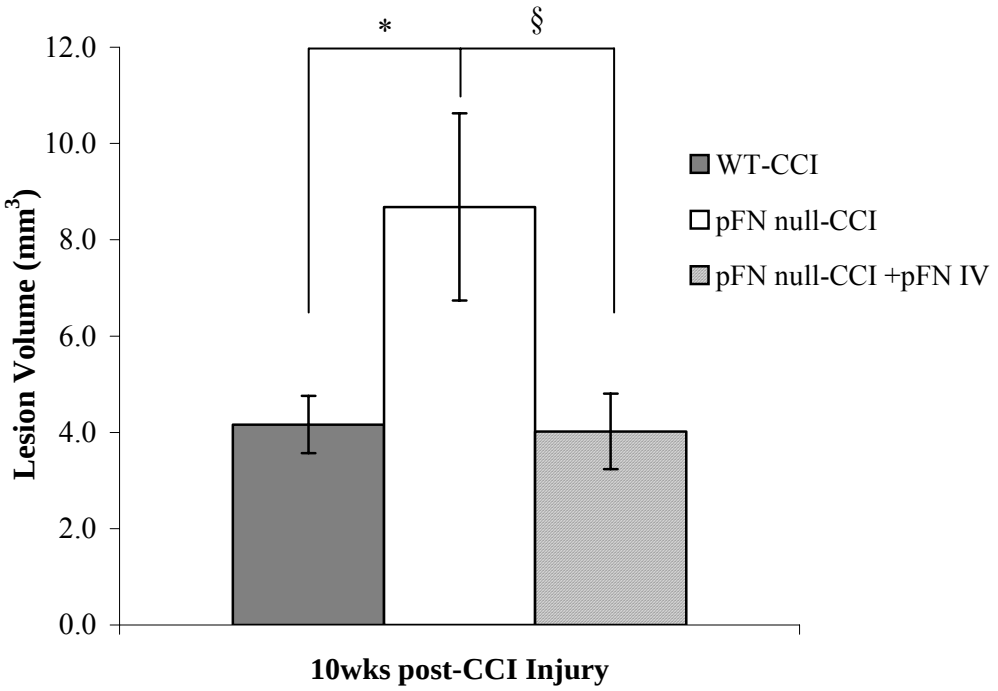

Figure 4.7: Lesion volume

At 10 weeks post-CCI injury, the lesion volume of injured mice deficient in pFN (pFN null-CCI) is significantly larger than injured WT mice (WT-CCI, *p=0.003) and pFN-null mice receiving an IV pFN injection (pFN null-CCI + pFN IV, §p=0.004). Mean ± SD

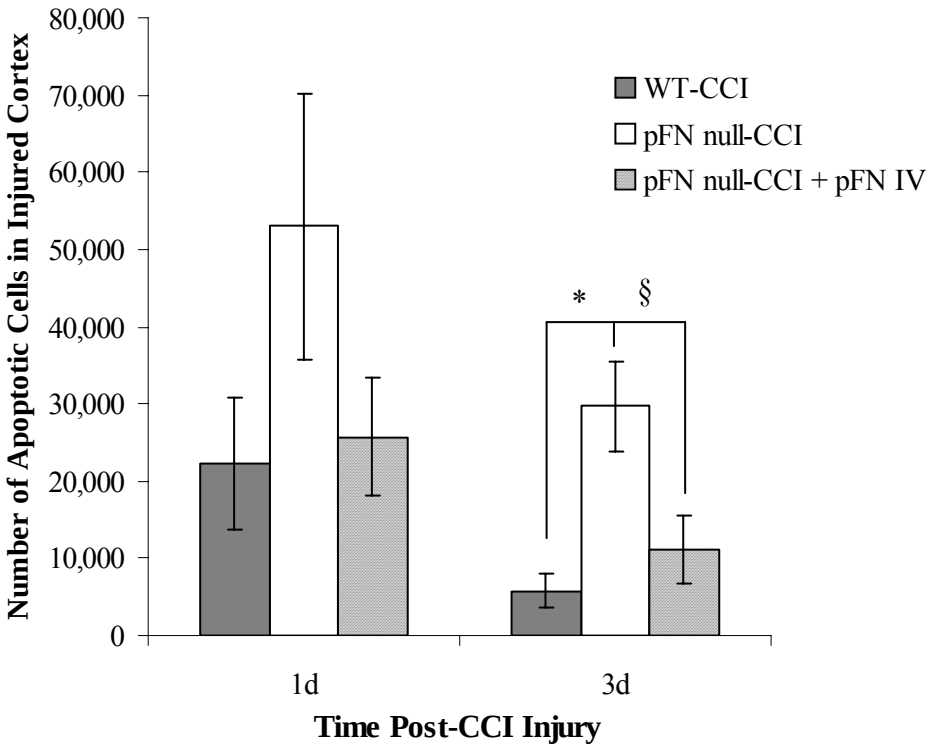

Figure 4.8: Apoptotic cell death in the injured cortex

At 3 days post-CCI injury, the amount of apoptotic cell death in the injured cortex for deficient in pFN (pFN null-CCI) is significantly larger than injured WT mice (WT-CCI, *p=0.002) and pFN-null mice receiving an IV pFN injection (pFN null-CCI + pFN IV, §p=0.028). Mean ± SD

Plasma fibronectin supports survival of astrocytes in vitro

While neurons bound to FN have been shown to have delayed apoptosis upon serum withdrawal in vitro, we investigated whether this is also true for astrocytes. Astrocytes plated on FN (n=4) had significantly less cell death 24 hours after serum withdrawal versus those plated on PLL (n=4, p=0.04) (**Figure 4.9**). It appeared that as cells underwent cell death, they became detached from the surface, and this detachment started at the periphery of the well, then moved towards the center. To better account for dead floating cells that had been rinsed away by the time of staining, we quantified the amount of cell death in the center of the well (micrographs in **Figure 4.9**) and again found that astrocytes plated on FN had significantly less cell death 24 hours after serum withdrawal versus those plated on PLL (p=0.001).

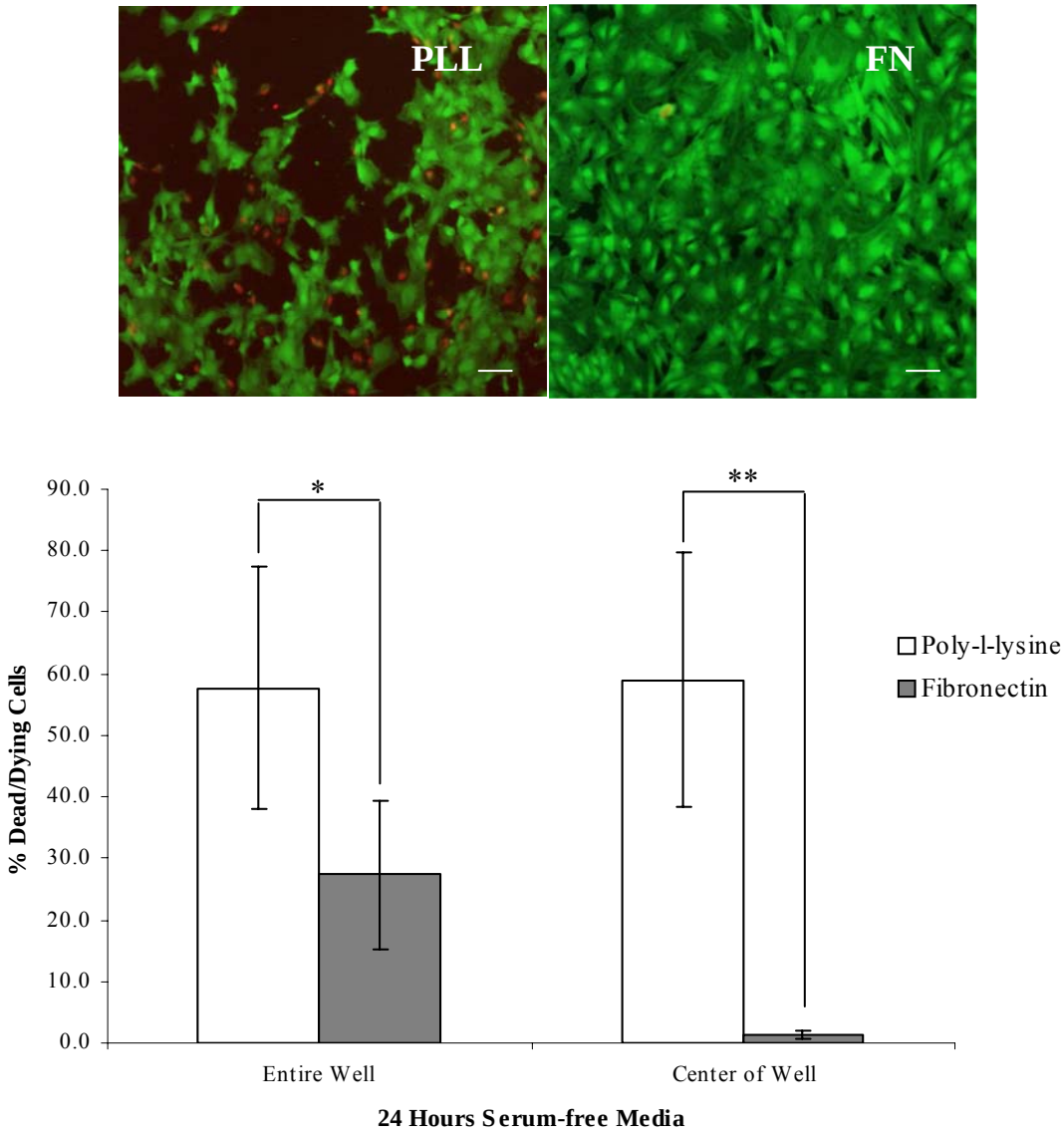

Figure 4.9: Fibronectin rescues astrocytes in vitro

Astrocytes plated on fibronectin had significantly less cell death 24 hours after serum withdrawal versus those plated on poly-l-lysine. Representative micrographs of the live (green) and dead/dying (red) cells remaining in the center of the well are shown above the plot. Scale bars = 100 μm; This data along with reports of similar trends for neurons indicate that direct binding of neural cells to fibronectin can prevent cell death. *p=0.04, **p=0.001, Mean ± SD

Plasma fibronectin null mice have decreased levels of phagocytic cells following brain injury

To determine if FN is involved in an inflammatory response after TBI similar to peripheral wound healing, the number of phagocytic cells was quantified. Cells that were both isolectin IB_4 positive and exhibited a rounded morphology were considered phagocytic cells, which could be either monocyte-derived macrophages or activated microglia, and were quantified using unbiased stereology. For all injured groups, these cells were present in blood clots at 3 days post-CCI injury and in the cortical injury penumbra at both 3 and 7 days post-CCI injury. For all the injured groups (pFN null, WT, pFN null + pFN IV), about half of the mice had blood clots at the injury site at 3 days, containing the large majority of phagocytic cells (likely blood macrophages), and there was no difference in the number of phagocytic cells between the injured groups at this time point ($p>0.8$, data not shown). Furthermore, Sakai et al. reported that pFN null and WT mice had the same number of macrophages in the injured brain 2 days following ischemia (Sakai et al., 2001). However, while the number of phagocytic cells decreases by 7 days post-CCI injury for all groups, there were significantly less macrophages / activated microglia in the injury periphery for the pFN null mice (10,930±8,800, n=4) compared to the WT littermates (45,920±22,680, n=5, $p=0.029$). Also, plasma FN null mice that received pFN IV had increased numbers of macrophages / activated microglia in the injury periphery (40,000±13,830, n=4, $p=0.083$) compared to mice without pFN (**Figure 4.10**). This suggests that while phagocytic cells are available at acute time points (primarily from the blood), the pFN may aid in recruiting or retaining phagocytic cells to the injured cortex, which underscores the role of FN in phagocyte adhesion, migration, and opsonization.

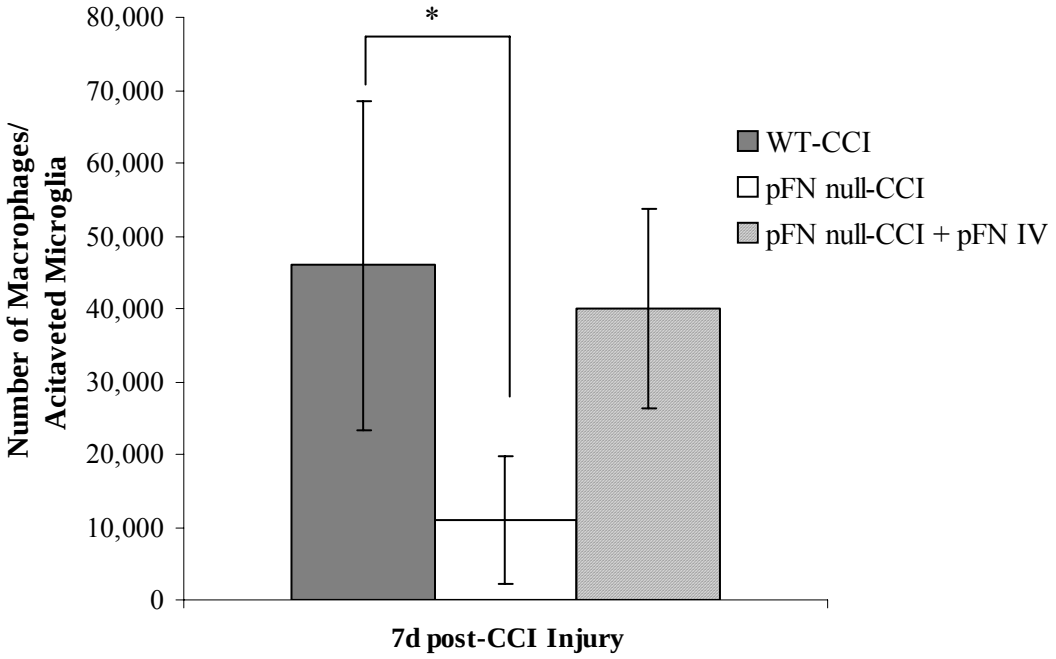

Figure 4.10: Phagocytic cells in the injured brain

At 7 days post-CCI, the number of phagocytic cells in the injured cortex for mice deficient in plasma FN (pFN null-CCI) is significantly lower than for injured WT mice (WT-CCI, *p=0.029), and the addition of plasma FN prior to injury seems to reverse this. Mean ± SD

Finally, to determine if FN is involved in forming or stabilizing the glial scar, we stained for glial fibrillary acidic protein (GFAP) to identify reactive astrocytes, which are the dominant cell type of the glial scar. We found no differences between the injured groups in either the density or morphology of $GFAP^+$ cells up to 7 days post-CCI injury (data not shown), indicating these cells are able to proliferate and migrate independent of pFN.

Discussion

We utilized conditional pFN knockout mice to investigate the role of pFN in TBI. Because the deletion of the *FN* gene in the liver occurs in adult animals, these mice develop normally and without the complications of compensatory mechanisms, such as increased expression of other ECM proteins (Sakai et al., 2001). We found that following experimental TBI, mice deficient in pFN had significantly worse motor and cognitive ability, significantly more cell death, and significantly less retention of phagocytic cells compared to WT mice. Furthermore, replacing the knocked out pFN (with a single IV injection of human pFN 6 hours prior to the injury) restored their behavior performance, cell death profiles, and levels of phagocytic cells to that of WT animals. The fact that the pFN replacement was able to rescue the knockout mice shows that these differences were due to specifically to pFN. Collectively, these data show that FN is neuroprotective to the traumatically injured brain.

While there were significant differences in apoptotic cell death, it is not clear if this is due to a direct binding of injured neural cells to pFN. In vitro studies of various cell types reveal that binding to FN via the $\alpha_5\beta_1$ integrin leads to anti-apoptotic signals including increases in Bcl-2 expression (Fukai et al., 1998; Zhang et al., 1995). For neural cell types, Gibson, et al., showed that neurons plated on FN had less apoptotic cell death after serum withdrawal, and FN induced anti-apoptotic signaling via $\alpha_5\beta_1$ integrin binding (Gibson et al., 2005). In addition, we found that pFN rescued astrocytes from

cell death after serum withdrawal. Together, this in vitro evidence shows that direct FN-integrin binding to neural cells initiates anti-apoptotic signaling. Both neurons and glial cells express $\alpha_5\beta_1$ in the adult brain (King et al., 2001), thus FN in the injured tissue may be binding directly to damaged neural cells to reduce apoptosis. Based on our findings alone, we cannot assess whether these cells are directly binding to the FN in the injured brain, or FN is helping the injured environment through an alternative mechanism which subsequently reduces cell death.

Another potential mechanism of protection for FN is through aiding in the inflammatory response to injury. Though the inflammatory response to brain injury is often considered detrimental, its beneficial roles are becoming more appreciated (Correale and Villa, 2004; Morganti-Kossmann et al., 2002; Schwartz, 2000). Furthermore, the use of progesterone (a promising clinical treatment for TBI) has been shown to improve neurological outcome in injured rats and is associated with an increase in macrophages / activated microglia (Grossman et al., 2004; Shear et al., 2002). In this study, we found that animals that had enhanced motor and cognitive deficits and increased cell death also had fewer phagocytic cells in the injury penumbra at 7 days post-injury. The role of phagocytes in initial clearance of injured tissue is certainly important in reducing the spread of cell death to neighboring tissue by clearing potentially toxic components of necrotic cells. Regarding the association of FN with macrophages / activated microglia, it is likely that FN is aiding in phagocytosis. The relationship between macrophages and FN has been studied to a large extent with respect to wound healing mechanisms (Clark, 1988; 1990; Clark et al., 1982; Grinnell, 1984; Martin et al., 1988; Reese et al., 1982; Tonnesen et al., 2000). Fibronectin is known to promote adhesion and migration of macrophages, and acts as an opsonin, particularly with gelatinous material, such as damaged tissue (Grinnell, 1984; Martin et al., 1988). Furthermore, binding of microglial cells to FN promotes adhesion, migration, and proliferation (Liao et al., 2005; Nasu-Tada

et al., 2005), and the activation of microglia is associated with elevated expression of integrins which bind FN (Milner and Campbell, 2003). Thus, FN may also support the recruitment and expansion of microglia in response to traumatic injury. Binding of macrophages to FN leads to the release of the pro-inflammatory cytokine TNFα (Hoyte et al., 1997), which has been shown to be beneficial to for long-term repair and recovery following experimental TBI (Scherbel et al., 1999), and binding of microglia to FN leads to increased production of the anti-inflammatory cytokine TGFβ, and nerve growth factor (Liao et al., 2005). Thus, FN may increase the production of neuroprotective or neurotrophic molecules in the injured brain tissue.

Since the absence of pFN led to increased impairment following TBI, a logical inference is that excess or super-physiologic levels of FN in the brain may enhance recovery. Furthermore, in experimental brain ischemia, IV administration of functional FN peptides led to reduced lesion size and improved neurological outcome (Yanaka et al., 1996; Zhao et al., 2005). However, the IV injections of pFN given in this study restored less than normal concentrations of FN in the blood; and we found that these mice were statistically the same as WT mice for the outcome measures assessed. Thus, the protective mechanisms involving FN may require less than physiologic levels. However, in examining the lesion size of animals from the study in Chapter 2, we found that mice receiving intracranial injections of the FN (100 μg/ml) and collagen I (1 mg/ml) scaffold at 7 days post-injury had significantly decreased lesion volume at 9 weeks post-injury (**Figure 4.11**). Note that these injections were given after the closing of the BBB and at a time when the amount of endogenous FN is approaching uninjured levels. Perhaps the presence of the FN in the injured brain at this time was able to promote survival of cells in the injury penumbra and reduce the ongoing spread of the lesion. Another potential explanation is that the scaffold encouraged migration of endogenous cells into the injury cavity. Additional studies involving administration of FN – either intravenously at acute

time points post-TBI (while the BBB is more permeable) or at sub-acute time points (either intracranially or in conjunction with re-opening the BBB) to maintain higher levels of FN in the injured brain – are warranted to address the potential use of FN in a treatment paradigm. In any case, a better understanding of the components and mechanisms involved in brain injury are critical steps to developing more effective treatment strategies. Moreover, the reparative/protective nature of FN in the brain may potentially be exploited for other neurological disorders that do not involve breaches in the BBB. Given the complex and dynamic microenvironment of the traumatically injured brain, it may be beneficial to use a multi-level treatment approach, such as administering FN in combination with cell transplantation.

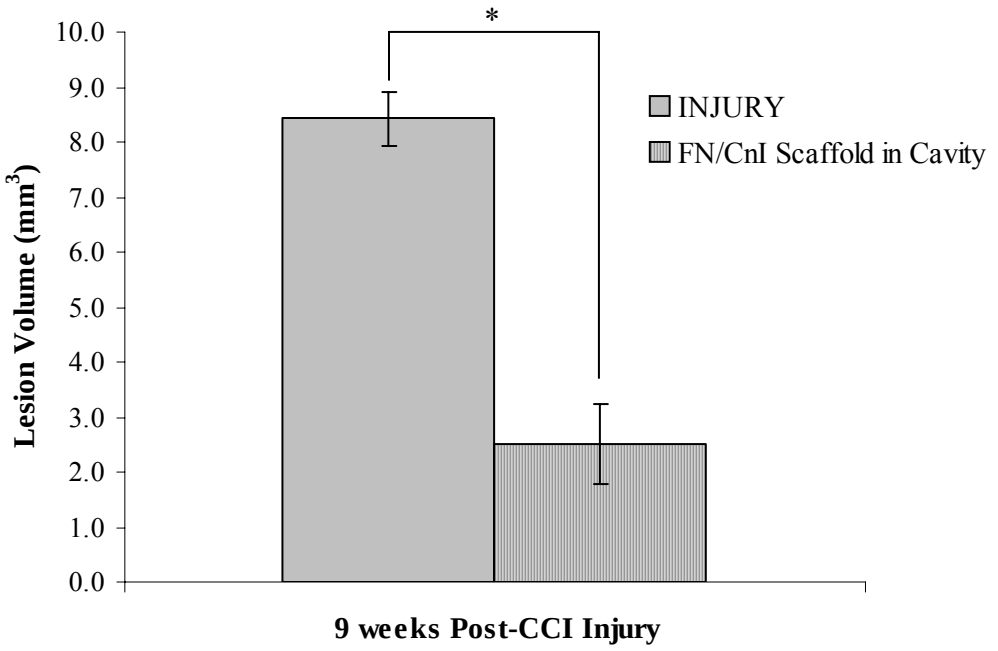

Figure 4.11 Lesion volumes following intracranial injection of fibronectin 1 week post- injury

Injured C57BL6 mice received intracranial injections of a scaffold containing FN (100 μg/ml) and collagen I (CnI; 1 mg/ml) at 1 week post-CCI injury. By 9 weeks post-CCI injury, treated animals had a significant reduction in lesion volume compared to untreated animals (*p=0.01).

The presence of FN in the injured brain has been identified by many groups following both clinical and experimental TBI. Since the majority of this FN is plasma borne, it was previously unclear if or how the brain was actively utilizing this protein. We have shown here that FN in the traumatically injured brain is protective against cell death, which ultimately correlates with behavioral function. Previous work by Sakai et al. showed that pFN is also protective following cerebral ischemia (Sakai et al., 2001). This indicates that when FN is available in the injured brain (i.e. there is a breach in the BBB), this protein is utilized in a reparative fashion. Therapeutic exploration of endogenous repair systems may be a promising strategy for improving outcome after brain injury.

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CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

Tissue Engineering Strategies Improve Cell Transplantation into the Injured Brain

Cell transplantation is an appealing treatment strategy for disorders of the central nervous system (CNS), and has already shown promise in the clinic for treating traumatic brain injury (TBI) (Seledtsov et al., 2005). Exploiting cells for transplantation offers huge potential, ranging from using these biologically active systems to produce and secrete specific neurotrophic factors to replacing lost cells. Advantages of transplanting cells include the ability to target multiple mechanisms and the ability to provide a sustained treatment. Furthermore, cells, particularly stem cells, can adapt to their environment, thus are able to evolve with the ever-changing pathologic brain. Using stem or progenitor cells also offers the opportunity to have either "off-the-shelf" treatments (e.g., renewable cell source such as embryonic stem cells) or autologous grafts (e.g., bone marrow-derived mesenchymal stem cells). Cell source is also important when considering the host immune response to the grafted cells. Again, autologous stem cells as well as embryonic stem cells (due to their relatively low levels of major histocompatibility complex) would be preferred, and work is currently underway in our laboratory, as well as others, to push these pluripotent stem cells towards neural phenotypes. While many groups are exploring various cell types for treating brain injury or disease, few focus on optimizing transplant conditions.

One major limitation to cell transplantation has been poor survival of donor cells in the injured or diseased brain. Our goal is to improve the survival and integration of donor cells in an effort to enhance recovery. Fetal tissue pieces grafted into the injured brain have shown promising results, likely because these are relatively stable grafts that are less

vulnerable to cell death and contain critical components that aid in repair. However, limitations to fetal tissue transplants include inadequate availability and ethical concerns, technical difficulties keeping tissue pieces viable in vitro, and potentially invasive delivery of a three-dimensional implant. These limitations can be overcome by engineering a tissue-like construct based on core components of the developing (fetal) brain tissue: neural stem cells (NSC), extracellular matrix (ECM) proteins, and in situ three-dimensionality. We expect that this is an improvement over cell suspension transplants due to both the ability to control the environment of the donor cells and the presence of components that may be crucial to graft success in rescuing damaged tissue.

In this work, we examined the effect of using a tissue engineered construct containing NSC plus an ECM protein-based scaffold on donor cell survival and integration, and cognitive behavior or treated animals following transplantation into the traumatically injured brain. The two ECM-based scaffolds investigated were fibronectin-collagen I (FN/CnI) and laminin-collagen I (LN/CnI). Fibronectin and LN were utilized because of their critical involvement in the developing brain. In addition to using proteins that support donor cells through cell-matrix signaling, we wanted the ability to deliver a tissue construct that was three-dimensional in situ, but could be delivered in a minimally invasive fashion. Thus, CnI was chosen for its thermally-reversible properties.

We found that both FN and LN containing scaffolds could be injected as a viscous liquid, and form a gel upon reaching physiologic temperatures, thus achieving the goal of a three-dimensional microenvironment for the donor cells with minimally invasive delivery. We also found that donor cells in both scaffolds examined had improved survival compared to cells transplanted alone. Interestingly, this effect was not observed at the early post-transplant time points examined (1 and 3 weeks), but it was evident by 8 weeks post-transplant. While initially donor cell survival in all groups was similar (~3-

5%), the rate of subsequent cell death over the next several weeks was slowed for the groups that received the tissue engineered scaffolds. This may be due to a number of reasons, including selection of a more robust cell type(s) during the very early post-transplant period or improving the condition of the host tissue, thus creating a more favorable environment for donor cell survival. From a clinical standpoint, it would be ideal to deliver this type of treatment with only a single injection, thus encouraging longer term graft survival will be crucial.

In comparing the two scaffolds examined, the LN/CnI scaffold was better at maintaining cell survival than the FN/CnI scaffold. Also, the group that was treated with the NSC+LN/CnI scaffold showed significant improvement in cognitive ability, while the other treatment groups only had a modest affect on this cognitive recovery. Though both FN and LN are critical to neural development, are increased after TBI, and support migration and adhesion of NSC in vitro, the LN-based scaffold was more advantageous through mechanisms that are not fully understood. This response may be partially explained in light of in vitro data demonstrating NSC had increased migration on LN compared to FN, which may result in enhanced integration in vivo with host tissue. However, no migration differences in the brain were observed between the treatment groups in the study presented here, underlying the complexities of the post-injury environment in vivo. Another difference that we previously observed in vitro is that NSC plated on LN had a slight, yet significant increase in differentiation into neuron-like cells. But, again this did not reflect the in vivo setting, as we did not identify any $NeuN^+$ donor cells. Furthermore, a higher percentage of neurons does not necessarily translate to better outcomes, as glial cells may provide sustained and sufficient neurotrophic support. In addition, it is unclear in any neural transplant setting (clinical or animal) whether transplanted neurons integrate into existing neuronal circuits in a functional manner. Future in vitro studies with LN and NSC and/or injured tissue may help identify

mechanisms by which LN improves survival of NSC. Our laboratory has developed an in vitro model of neural injury which consists of neurons and astrocytes cultured three-dimensionally in Matrigel®. Efforts are underway to use this as a test bed for various transplant conditions (Cullen et al., In preparation 2006), though the use of Matrigel® complicates studies with LN and alternatives are being considered. As far as future work with applying tissue engineering strategies for TBI, the use of a LN-based scaffold is recommended.

Given the technical difficulties associated with our tissue engineering work, it might be necessary to make a case for using tissue engineering in our model of TBI at all, especially since transplanting NSC into the striatum also promotes functional recovery. Addressing the technical issues first, these are largely due to the fact that we are injecting viscous materials into a very small cavity. The volume we deliver is based on volume estimates of the cavity made using histological sections. In a live animal, however, the "cavity" is sometimes filled with fluid, making the injection more difficult, and increasing variability. Work in higher-order mammals (with larger injuries) may alleviate some of these issues, though careful consideration should be given to injection volumes into damaged tissue. As far as the promising results we have had with striatal injections, this may be due to the fact that a common feature of both transplant paradigms was an extracellular support for transplanted cells, whether it was from native striatal tissue or a scaffold co-delivered with the cells. From a clinical standpoint, it is less invasive to transplant into cortical areas versus deeper brain structures, and transplanting into non-injured tissue distal to the injury should be avoided if transplants at the site of injury are comparably effective. Though clinical brain injuries have much more widespread damage than the focal injuries modeled experimentally, cortical injury is a common feature seen in blunt (non-penetrating) trauma. In the work presented here we delivered construct volumes that would potentially "fill" the injury cavity. However, due

to the increased scale and scope of clinical TBI, this may be difficult, requiring large amounts of both cells and scaffold materials. However, our aim is to control the *microenvironment* of the donor cells. Thus, using small volumes will still be appropriate. Perhaps at the time of transplant, multiple injections could be delivered proximal to the injury site(s). These types of considerations are also important when expanding tissue engineering techniques to other CNS injuries and diseases. Tissue engineering strategies increase donor cell survival and can be exploited to augment cell transplantation for many disorders, including spinal cord injury, ischemia, and Parkinson's disease. Our work in experimental TBI can be considered a representative (though extremely complex) in vivo model for identifying candidate scaffolds (and cells) for use in a tissue engineered treatment approach.

To this end, further investigation is warranted that may address some of the questions raised by the work presented here. Firstly, it cannot be ignored that a significant amount of donor cell death occurs within the first week following transplantation. Obviously, since low percentages of surviving cells are seen in multiple transplant settings, a reasonable strategy may be to transplant more cells. Furthermore, it has been demonstrated that increasing the number of cells transplanted actually improves the percentage that will survive (Saporta et al., 1999). However, transplanting more cells is a potentially costly solution, is limited from a technical standpoint, and may not be feasible when using autologous cell sources; thus demanding efforts to maximize the percentage of survival cells in other ways. Elucidating some of the mechanisms governing this acute cell loss is necessary (e.g., with the in vitro test bed described above) and may lead to engineering a scaffold that actively reduces the observed cell death. Along the lines of more precisely engineering scaffolds, using polymers, such as methylcellulose, may provide more control over specific design criteria and could be tailored to direct cell function. Though we may not currently know enough about the needs of the injured

brain (which is partly the motivation for using multipotential cells), as more information regarding successful transplants becomes available, having a moldable scaffold design will be beneficial. Additionally, the next phase of this work will also need to address the use of alternate cell sources that are more clinically feasible, such as embryonic stem cell lines or bone marrow-derived stem cells. To summarize, we have made significant progress by using tissue engineering to improve cell transplantation therapy for TBI. We need to address issues about how best to further enhance cell survival and integration, partially with the use of in vitro models, and utilize this information to improve our tissue engineered construct design.

Fibronectin and Laminin are Involved in Host Response to Brain Injury

In addition to investigating how the host environment influences donor cells, it is important to understand how ECM-based scaffolds affect the host environment. Though we intended to exploit these proteins for their ability to affect NSC function, FN and LN may also directly benefit the injured brain. Both FN and LN are involved in neural development, and these proteins are found in increased levels following brain injury. Furthermore, the brain may be utilizing these proteins in a reparative or regenerative fashion after injury. Thus, utilizing FN and LN could both directly improve survival and function of donor cells and augment endogenous repair mechanisms, thereby further contributing to an environment within the host brain that is more amenable to donor cells.

While these proteins had been found in increased levels following clinical and experimental TBI, their presence had not been examined using an experimental model of blunt trauma, which is the most common type of clinical TBI. Since we were interested in investigating these proteins in such a model, we needed to first establish the distribution pattern in injured brains using an in vivo model of blunt trauma. Thus, we investigated the spatiotemporal profile of FN and LN following controlled cortical impact

(CCI) injury. Increased FN^+ and LN^+ tissue was observed up to 14 days post-CCI injury, and the levels were significantly higher than sham controls at 3 days post-CCI injury. The FN^+ staining appeared in the extracellular tissue adjacent to the injury, including blood clots at the site of impact found at acute time points. The LN^+ staining was primarily located in blood vessels in the tissue adjacent to the injury.

We also wanted to determine if FN and LN correlated with support cells to begin to elucidate potential roles for these proteins in the injured brain. We found that FN^+ tissue was populated with macrophages and/or activated microglia, indicating a role for FN in aiding in the functions of these phagocytic cells. Also, reactive astrocyte processes were found wrapped around the majority of LN^+ vessels, implying LN may be involved in angiogenesis and/or reformation of the blood-brain barrier (BBB).

The co-spatialization of the increased ECM proteins with supporting cells warrants further investigation into their hypothesized beneficial role in the injured brain. For example, the tight spatial correlation of LN^+ vessels with reactive astrocytes we observed and the correlation of the time course of the increases in LN with the biphasic opening of the BBB (Baskaya et al., 1997) imply a role in BBB repair or reformation. Further experiments should be conducted which alter the amount of LN in the injured brain and investigate the resulting effects on factors related to re-establishing the vasculature and BBB (e.g., angiogenic activity and BBB permeability). While these types of experiments will not provide direct causality information, they address specific functions of LN in the injured brain. The effects of LN on BBB function after injury could be examined by providing supernormal levels of LN to the injured brain (via either intravenous or intracranial administration). Alternatively, the amount of available LN in the injured brain could be decreased by reducing expression of LN RNA with short interference RNA or by blocking LN specific integrin dimers (e.g., $\alpha_6\beta_1$) to prevent LN from binding

to cells. However, both of these options have limitations, including inefficient deletion of LN or LN-integrin binding (particularly in vivo) and potentially disturbing other molecules, cells, or pathways, careful interpretation of the results. An elegant way to study the role of LN would be with a conditional LN knockout; however, this transgenic animal is not currently available. On the other hand, a conditional FN knockout is available, which allowed us to further examine the role of this ECM protein in the injured brain.

To summarize this work, we evaluated the spatiotemporal profile of two ECM proteins involved in neural development. We were the first to evaluate these proteins using a model of blunt trauma, which is the most common type of clinical TBI, and is a widely used as an experimental model. We also propose potential mechanisms of action for the increased amounts of FN and LN based on spatial localization with cells and integrating with previous evidence from the literature. The role of FN in TBI was subsequently further investigated.

Fibronectin is Neuroprotective in Traumatic Brain Injury

Fibronectin has been found to increase in the traumatically injured brain, though its functions were not known. Furthermore, since the majority of FN enters the brain from the blood (during breaches in the BBB), it was not known if the protein was actively contributing to the injury response. We investigated for the first time the role of FN in the traumatically injured brain utilizing adult, conditional plasma FN knockout (pFN null) mice. We found that injured pFN null, when mice compared to injured wild type (WT) mice, had significantly more severe motor and cognitive deficits, significantly more apoptotic cell death at acute time points, significantly larger lesion volumes by 10 weeks post-injury, and significantly less phagocytic cells in the injured cortex at 1 week post-injury. Furthermore, a single intravenous injection of pFN into pFN null mice prior

to injury restored the above functions to that of injured WT mice. These results indicate that FN is neuroprotective to the traumatically injured brain.

Based on our findings, two potential mechanisms are proposed for how FN is beneficial in the injured brain. Firstly, FN could be providing an anti-apoptotic signal for damaged neural cells. Previous in vitro work has shown that FN is anti-apoptotic for several cell types, including neurons, and that binding of FN to $\alpha_5\beta_1$ integrins leads to increased production of the anti-apoptotic molecule Bcl-2. Here we showed that FN also promotes the in vitro survival astrocytes, which outnumber neurons 10:1, and whose function in the brain is an important part of normal and restorative physiology. In injured pFN null mice, we observed significantly more apoptotic cell death at acute time points and lesions that were twice as large compared to injured mice that do not lack FN. Since neurons and glial both express the $\alpha_5\beta_1$ integrins in the adult brain (though FN is essentially absent in the normal adult brain), these cells may be binding to FN after injury. Thus, FN may provide a direct survival (i.e. anti-apoptotic) signal to cells in the injury penumbra.

A second potential mechanism identified in this work is that FN promotes phagocytosis of dead tissue, thus helping to reduce the spread of cell death due to the toxic environment created by necrosis. We found that FN is involved in retaining phagocytic cells in the tissue bordering the lesion. This observation was noted at one week post-injury which is after the majority of the initial clearance occurs. Due to the fact that several of the injured animals had blood clots, we could not tease out differences in phagocytic cells in more acute time points. However it is likely FN is being exploited by phagocytic cells at the early time points given the evidence for FN in supporting the adhesion, migration and proliferation of both macrophages and microglia, and the role of FN as an opsonin for the removal of tissue debris. In addition, FN may also have a role in later time points after injury since FN promotes secretion of beneficial factors in

microglia, which may aid in repair or regeneration. These processes also promote cell survival, indicating FN may decrease apoptosis via indirect pathways. A combination of these mechanisms likely accounts for the striking differences between injured mice having or lacking pFN.

We have demonstrated that FN is neuroprotective, and specifically contributes to cell survival and retention of phagocytic cells following experimental TBI. We have presented potential processes occurring at the cellular and molecular levels, however further investigation at the intracellular level can further direct treatment strategies. Focusing on the role of FN with phagocytic cells there are three main potential avenues of action requiring further investigation: 1- FN promotes migration, adhesion and/or proliferation of macrophages and/or microglia, 2- FN acts as an opsonin, and 3- FN increases secretion of neurotrophic factors. The next phase of this work will begin to address these potential actions, and the conditional pFN knockout could again be exploited to test all of these hypotheses. For example, to determine if FN promotes opsonization in the injured brain, we can measure the extent to which phagocytic cells are engaging in phagocytosis in injured pFN null mice versus wild type mice. Since active phagocytosis requires increased alterations to the cytoskeleton, this could be identified with a marker that specifically binds to filamentous actin (e.g., phallacidin). In addition, investigating these events in vitro may help identify specific mechanisms without the complications of competing factors and processes that arise in vivo. And, isolating specific signaling molecules may be more straightforward with in vitro preparations. While all three of the aforementioned roles for FN with phagocytic cells have been examined in vitro, this has not been done in the presence of injured brain tissue, which has unique properties. For example, if cultured macrophages engulf injured neural tissue or cells in vitro, we can investigate if this is enhanced by the addition of FN, and begin to elucidate the specific signaling molecules involved.

Given the fact that we determined pFN is neuroprotective in TBI and previous work with conditional pFN knockout mice revealed it is also neuroprotective in cerebral ischemia, a logical assumption is that FN may be beneficial in a treatment paradigm. Indeed, intravenous injections of functional FN peptides have been found to be beneficial in experimental cerebral ischemia. In the work presented here, the recovery of injured mice with sub-physiologic levels of FN was comparable to injured mice with normal levels for the outcome measures we examined. This indicates that the system may be saturated, and super-normal levels may not be effectively utilized by injured brain tissue. Future work with this project includes delivering FN (protein or peptides) to wild type animals at acute time points following TBI via intravenous injections, thus allowing the FN protein or peptides to enter via the normal route during an increased permeability of the BBB. However, it may be more beneficial to provide FN at sub-acute time points, when the endogenous levels have decreased towards normal amounts. We have shown that injured wild type mice that received an intracranial injection of a FN-containing scaffold at 1 week post-injury had significantly decreased lesion volume compared to untreated mice. Since intracranial delivery is more invasive than intravenous routes, FN could also be delivered at post-injury time points when the BBB has already resealed by exploiting agents that temporarily increase BBB permeability. This technique could also be used to provide FN to the brain in disorders that are not associated with opening of the BBB. Future work for this project will include administering FN protein or peptides after endogenous post-TBI levels have been restored to normal in an expanded effort to examine the potential for FN in a treatment paradigm. Moreover, studies investigating super-physiologic amounts of FN also increase our understanding of the relationship of FN with injured brain tissue. A better appreciation of the injury response at multiple levels can improve treatment efforts, including combinatorial therapies, to repair the injured brain and enhance functional recovery of brain injured patients.

In conclusion, we determined that FN is neuroprotective to the injured brain; specifically FN decreases cell death and increases the presence of phagocytic cells. Elucidating these mechanisms contributes to a better understanding of the complex pathology of TBI, which ultimately improves treatment strategies. In light of the complicated and evolving endogenous response to TBI, it is likely that multi-targeted treatment strategies will be required. One promising treatment strategy is cell transplantation, partially because it utilizes biologically active, adaptable systems that can respond to the needs of the injured brain. We have showed that we can improve the survival of NSC transplanted into the injured brain by supporting the cells with a FN or LN based scaffold, and the NSC co-transplanted within a LN scaffold enhance cognitive recovery. Improving survival and integration of transplanted cells is a critical step for treating many disorders and diseases of the CNS. The dissertation work presented here has broad applications, benefiting the fields of brain injury, ECM biology, cell transplantation, and neural tissue engineering.

REFERENCES

Akimov, S. S. and Belkin, A. M. (2001) Cell surface tissue transglutaminase is involved in adhesion and migration of monocytic cells on fibronectin. *Blood* **98,** 1567-76.

Akiyama, S. K. (1996) Integrins in cell adhesion and signaling. *Hum Cell* **9,** 181-6.

Aota, S., Nomizu, M. and Yamada, K. M. (1994) The short amino acid sequence Pro-His-Ser-Arg-Asn in human fibronectin enhances cell-adhesive function. *J Biol Chem* **269,** 24756-61.

Aumailley, M., Bruckner-Tuderman, L., Carter, W. G., Deutzmann, R., Edgar, D., Ekblom, P., Engel, J., Engvall, E., Hohenester, E., Jones, J. C., Kleinman, H. K., Marinkovich, M. P., Martin, G. R., Mayer, U., Meneguzzi, G., Miner, J. H., Miyazaki, K., Patarroyo, M., Paulsson, M., Quaranta, V., Sanes, J. R., Sasaki, T., Sekiguchi, K., Sorokin, L. M., Talts, J. F., Tryggvason, K., Uitto, J., Virtanen, I., von der Mark, K., Wewer, U. M., Yamada, Y. and Yurchenco, P. D. (2005) A simplified laminin nomenclature. *Matrix Biol* **24,** 326-32.

Baehrecke, E. H. (2005) Autophagy: dual roles in life and death? *Nat Rev Mol Cell Biol* **6,** 505-10.

Barker, R. A., Dunnett, S. B., Faissner, A. and Fawcett, J. W. (1996) The time course of loss of dopaminergic neurons and the gliotic reaction surrounding grafts of embryonic mesencephalon to the striatum. *Exp Neurol* **141,** 79-93.

Baskaya, M. K., Rao, A. M., Dogan, A., Donaldson, D. and Dempsey, R. J. (1997) The biphasic opening of the blood-brain barrier in the cortex and hippocampus after traumatic brain injury in rats. *Neurosci Lett* **226,** 33-6.

Bellail, A. C., Hunter, S. B., Brat, D. J., Tan, C. and Van Meir, E. G. (2004) Microregional extracellular matrix heterogeneity in brain modulates glioma cell invasion. *Int J Biochem Cell Biol* **36,** 1046-69.

Bi, X., Lynch, G., Zhou, J. and Gall, C. M. (2001) Polarized distribution of alpha5 integrin in dendrites of hippocampal and cortical neurons. *J Comp Neurol* **435,** 184-93.

Cernak, I. (2005) Animal models of head trauma. *NeuroRx* **2,** 410-22.

Chen, Z. L., Indyk, J. A. and Strickland, S. (2003) The hippocampal laminin matrix is dynamic and critical for neuronal survival. *Mol Biol Cell* **14,** 2665-76.

Clark, R. A. (1988) Potential roles of fibronectin in cutaneous wound repair. *Arch Dermatol* **124,** 201-6.

Clark, R. A. (1990) Fibronectin matrix deposition and fibronectin receptor expression in healing and normal skin. *J Invest Dermatol* **94,** 128S-134S.

Clark, R. A., Quinn, J. H., Winn, H. J., Lanigan, J. M., Dellepella, P. and Colvin, R. B. (1982) Fibronectin is produced by blood vessels in response to injury. *J Exp Med* **156,** 646-51.

Clarkson, E. D., Zawada, W. M., Adams, F. S., Bell, K. P. and Freed, C. R. (1998) Strands of embryonic mesencephalic tissue show greater dopamine neuron survival and better behavioral improvement than cell suspensions after transplantation in parkinsonian rats. *Brain Res* **806,** 60-8.

Colognato, H. and Yurchenco, P. D. (2000) Form and function: The laminin family of heterotrimers. *Developmental Dynamics* **218,** 213-234.

Correale, J. and Villa, A. (2004) The neuroprotective role of inflammation in nervous system injuries. *J Neurol* **251,** 1304-16.

Cullen, D. K., Stabenfeldt, S. E., Simon, C. M., Tate, C. C., Schumm, M. A. and LaPlaca, M. C. (In preparation 2006) A surrogate injury environment to optimize neural stem cell construct delivery.

Danen, E. H. and Yamada, K. M. (2001) Fibronectin, integrins, and growth control. *J Cell Physiol* **189,** 1-13.

Dash, P. K., Mach, S. A. and Moore, A. N. (2001) Enhanced neurogenesis in the rodent hippocampus following traumatic brain injury. *J Neurosci Res* **63,** 313-9.

David, S. and Aguayo, A. J. (1981) Axonal elongation into peripheral nervous system "bridges" after central nervous system injury in adult rats. *Science* **214,** 931-3.

Davis, G. E. and Senger, D. R. (2005) Endothelial extracellular matrix: biosynthesis, remodeling, and functions during vascular morphogenesis and neovessel stabilization. *Circ Res* **97,** 1093-107.

Dixon, C. E., Clifton, G. L., Lighthall, J. W., Yaghmai, A. A. and Hayes, R. L. (1991) A controlled cortical impact model of traumatic brain injury in the rat. *J Neurosci Methods* **39,** 253-62.

Egan, R. A. and Vijayan, V. K. (1991) Fibronectin immunoreactivity in neural trauma. *Brain Res* **568,** 330-4.

Einheber, S., Schnapp, L. M., Salzer, J. L., Cappiello, Z. B. and Milner, T. A. (1996) Regional and ultrastructural distribution of the alpha 8 integrin subunit in developing and adult rat brain suggests a role in synaptic function. *J Comp Neurol* **370,** 105-34.

Fox, G. B., Fan, L., Levasseur, R. A. and Faden, A. I. (1998) Sustained sensory/motor and cognitive deficits with neuronal apoptosis following controlled cortical impact brain injury in the mouse. *J Neurotrauma* **15,** 599-614.

Fox, G. B., LeVasseur, R. A. and Faden, A. I. (1999) Behavioral responses of C57BL/6, FVB/N, and 129/SvEMS mouse strains to traumatic brain injury: implications for gene targeting approaches to neurotrauma. *J Neurotrauma* **16,** 377-89.

Freire, E., Gomes, F. C., Jotha-Mattos, T., Neto, V. M., Silva Filho, F. C. and Coelho-Sampaio, T. (2004) Sialic acid residues on astrocytes regulate neuritogenesis by controlling the assembly of laminin matrices. *J Cell Sci* **117,** 4067-76.

Fukai, F., Mashimo, M., Akiyama, K., Goto, T., Tanuma, S. and Katayama, T. (1998) Modulation of apoptotic cell death by extracellular matrix proteins and a fibronectin-derived antiadhesive peptide. *Exp Cell Res* **242,** 92-9.

Gauperaa, T. and Seljelid, R. (1985) Plasma fibronectin contributes to fibronectin in tissues. *Acta Chir Scand* **151,** 193-9.

George, E. L., Georges-Labouesse, E. N., Patel-King, R. S., Rayburn, H. and Hynes, R. O. (1993) Defects in mesoderm, neural tube and vascular development in mouse embryos lacking fibronectin. *Development* **119,** 1079-91.

Gibson, R. M. (1999) Caspase activation is downstream of commitment to apoptosis of Ntera-2 neuronal cells. *Exp Cell Res* **251,** 203-12.

Gibson, R. M., Craig, S. E., Heenan, L., Tournier, C. and Humphries, M. J. (2005) Activation of integrin alpha5beta1 delays apoptosis of Ntera2 neuronal cells. *Mol Cell Neurosci* **28,** 588-98.

Giulian, D., Chen, J., Ingeman, J. E., George, J. K. and Noponen, M. (1989) The role of mononuclear phagocytes in wound healing after traumatic injury to adult mammalian brain. *J Neurosci* **9,** 4416-29.

Grant, D. S., Kleinman, H. K. and Martin, G. R. (1990) The role of basement membranes in vascular development. *Ann N Y Acad Sci* **588,** 61-72.

Grinnell, F. (1984) Fibronectin and wound healing. *J Cell Biochem* **26,** 107-16.

Grossman, K. J., Goss, C. W. and Stein, D. G. (2004) Effects of progesterone on the inflammatory response to brain injury in the rat. *Brain Res* **1008,** 29-39.

Gundersen, H. J. (1986) Stereology of arbitrary particles. A review of unbiased number and size estimators and the presentation of some new ones, in memory of William R. Thompson. *J Microsc* **143 (Pt 1),** 3-45.

Hagg, T., Portera-Cailliau, C., Jucker, M. and Engvall, E. (1997) Laminins of the adult mammalian CNS; laminin-alpha2 (merosin M-) chain immunoreactivity is associated with neuronal processes. *Brain Res* **764,** 17-27.

Hausmann, R. and Betz, P. (2000) The time course of the vascular response to human brain injury--an immunohistochemical study. *Int J Legal Med* **113,** 288-92.

Hauwel, M., Furon, E., Canova, C., Griffiths, M., Neal, J. and Gasque, P. (2005) Innate (inherent) control of brain infection, brain inflammation and brain repair: the role of microglia, astrocytes, "protective" glial stem cells and stromal ependymal cells. *Brain Res Brain Res Rev* **48,** 220-33.

Henderson, D. J. and Copp, A. J. (1997) Role of the extracellular matrix in neural crest cell migration. *J Anat* **191 (Pt 4),** 507-15.

Hertel, M., Tretter, Y., Alzheimer, C. and Werner, S. (2000) Connective tissue growth factor: a novel player in tissue reorganization after brain injury? *Eur J Neurosci* **12,** 376-80.

Holmin, S., Soderlund, J., Biberfeld, P. and Mathiesen, T. (1998) Intracerebral inflammation after human brain contusion. *Neurosurgery* **42,** 291-8; discussion 298-9.

Hoovler, D. W. and Wrathall, J. R. (1991) Implantation of neuronal suspensions into contusive injury sites in the adult rat spinal cord. *Acta Neuropathol* **81,** 303-11.

Hou, S., Xu, Q., Tian, W., Cui, F., Cai, Q., Ma, J. and Lee, I. S. (2005) The repair of brain lesion by implantation of hyaluronic acid hydrogels modified with laminin. *J Neurosci Methods* **148,** 60-70.

Hovda, D. A., Lee, S. M., Smith, M. L., Von Stuck, S., Bergsneider, M., Kelly, D., Shalmon, E., Martin, N., Caron, M., Mazziotta, J. and et al. (1995) The neurochemical and metabolic cascade following brain injury: moving from animal models to man. *J Neurotrauma* **12,** 903-6.

Hoyte, J. S., Standing, J. E. and Limper, A. H. (1997) Steady-state effects of vitronectin and fibronectin on the binding, uptake, and degradation of Pneumocystis carinii in rat alveolar macrophages. *Inflammation* **21,** 335-45.

Ikonomidou, C. and Turski, L. (2002) Why did NMDA receptor antagonists fail clinical trials for stroke and traumatic brain injury? *Lancet Neurol* **1,** 383-6.

Indyk, J. A., Chen, Z. L., Tsirka, S. E. and Strickland, S. (2003) Laminin chain expression suggests that laminin-10 is a major isoform in the mouse hippocampus and is degraded by the tissue plasminogen activator/plasmin protease cascade during excitotoxic injury. *Neuroscience* **116,** 359-71.

Johansson, C. B., Svensson, M., Wallstedt, L., Janson, A. M. and Frisen, J. (1999) Neural stem cells in the adult human brain. *Exp Cell Res* **253,** 733-6.

Kakinuma, Y., Hama, H., Sugiyama, F., Yagami, K., Goto, K., Murakami, K. and Fukamizu, A. (1998) Impaired blood-brain barrier function in angiotensinogen-deficient mice. *Nat Med* **4,** 1078-80.

Kao, W. J., Lee, D., Schense, J. C. and Hubbell, J. A. (2001) Fibronectin modulates macrophage adhesion and FBGC formation: the role of RGD, PHSRN, and PRRARV domains. *J Biomed Mater Res* **55,** 79-88.

Kato, H. and Walz, W. (2000) The initiation of the microglial response. *Brain Pathol* **10,** 137-43.

Kernie, S. G., Erwin, T. M. and Parada, L. F. (2001) Brain remodeling due to neuronal and astrocytic proliferation after controlled cortical injury in mice. *J Neurosci Res* **66,** 317-26.

Kiener, J. L., Saba, T. M., Cho, E. and Blumenstock, F. A. (1986) Clearance and tissue distribution of fibronectin in septic rats: relationship to synthetic rate. *Am J Physiol* **251,** R724-34.

King, V. R., McBride, A. and Priestley, J. V. (2001) Immunohistochemical expression of the alpha5 integrin subunit in the normal adult rat central nervous system. *J Neurocytol* **30,** 243-52.

Kobayashi, K., Haung, C. and Lodge, T. (1999) Thermoreversible Gelation of Aqueous Methylcellulous Solutions. *Macromolecules* **32,** 7070-77.

Kornblihtt, A. R., Umezawa, K., Vibe-Pedersen, K. and Baralle, F. E. (1985) Primary structure of human fibronectin: differential splicing may generate at least 10 polypeptides from a single gene. *Embo J* **4,** 1755-9.

Koutouzis, T. K., Emerich, D. F., Borlongan, C. V., Freeman, T. B., Cahill, D. W. and Sanberg, P. R. (1994) Cell transplantation for central nervous system disorders. *Crit Rev Neurobiol* **8,** 125-62.

Kulkarni, G. V. and McCulloch, C. A. (1994) Serum deprivation induces apoptotic cell death in a subset of Balb/c 3T3 fibroblasts. *J Cell Sci* **107 (Pt 5),** 1169-79.

La Celle, P., Blumenstock, F. A., McKinley, C., Saba, T. M., Vincent, P. A. and Gray, V. (1990) Blood-borne collagenous debris complexes with plasma fibronectin after thermal injury. *Blood* **75,** 470-8.

Labbe, R., Firl, A., Jr., Mufson, E. J. and Stein, D. G. (1983) Fetal brain transplant: reduction of cognitive deficits in rats with frontal cortex lesions. *Science* **221,** 470-2.

Ladeby, R., Wirenfeldt, M., Garcia-Ovejero, D., Fenger, C., Dissing-Olesen, L., Dalmau, I. and Finsen, B. (2005) Microglial cell population dynamics in the injured adult central nervous system. *Brain Res Brain Res Rev* **48,** 196-206.

Langlios, J., Rutland-Brown, W, Thomas KE (2004) Traumatic Brain Injury in the United States: Emergency Department Visits, Hospitalizations, and Deaths. Centers for Disease Control and Prevention, National Center for Injury Prevention and Control: Atlanta, GA.

Lariviere, B., Rouleau, M., Picard, S. and Beaulieu, A. D. (2003) Human plasma fibronectin potentiates the mitogenic activity of platelet-derived growth factor and complements its wound healing effects. *Wound Repair Regen* **11,** 79-89.

Lenzlinger, P. M., Morganti-Kossmann, M. C., Laurer, H. L. and McIntosh, T. K. (2001) The duality of the inflammatory response to traumatic brain injury. *Mol Neurobiol* **24,** 169-81.

Leone, D. P., Relvas, J. B., Campos, L. S., Hemmi, S., Brakebusch, C., Fassler, R., Ffrench-Constant, C. and Suter, U. (2005) Regulation of neural progenitor proliferation and survival by beta1 integrins. *J Cell Sci* **118,** 2589-99.

Liao, H., Bu, W. Y., Wang, T. H., Ahmed, S. and Xiao, Z. C. (2005) Tenascin-R plays a role in neuroprotection via its distinct domains that coordinate to modulate the microglia function. *J Biol Chem* **280,** 8316-23.

Liesi, P. (1992) Neuronal migration on laminin involves neuronal contact formation followed by nuclear movement inside a preformed process. *Exp Neurol* **117,** 103-13.

Lindvall, O. (2003) Stem cells for cell therapy in Parkinson's disease. *Pharmacol Res* **47,** 279-87.

Liu, H. M. and Sturner, W. Q. (1988) Extravasation of plasma proteins in brain trauma. *Forensic Sci Int* **38,** 285-95.

Maddox, D. E., Shibata, S. and Goldstein, I. J. (1982) Stimulated macrophages express a new glycoprotein receptor reactive with Griffonia simplicifolia I-B4 isolectin. *Proc Natl Acad Sci U S A* **79,** 166-70.

Maenpaa, A., Kovanen, P. E., Paetau, A., Jaaskelainen, J. and Timonen, T. (1997) Lymphocyte adhesion molecule ligands and extracellular matrix proteins in gliomas and normal brain: expression of VCAM-1 in gliomas. *Acta Neuropathol (Berl)* **94,** 216-25.

Martin, D. E., Reece, M. C., Maher, J. E. and Reese, A. C. (1988) Tissue debris at the injury site is coated by plasma fibronectin and subsequently removed by tissue macrophages. *Arch Dermatol* **124,** 226-9.

Matter, M. L. and Ruoslahti, E. (2001) A signaling pathway from the alpha5beta1 and alpha(v)beta3 integrins that elevates bcl-2 transcription. *J Biol Chem* **276,** 27757-63.

Max, W., MacKenzie, E. and Rice, D. (1991) Head injuries: costs and consequences. *J Head Trauma Rehab* **6,** 76-91.

McIntosh, T. K., Saatman, K. E., Raghupathi, R., Graham, D. I., Smith, D. H., Lee, V. M. and Trojanowski, J. Q. (1998) The Dorothy Russell Memorial Lecture. The molecular and cellular sequelae of experimental traumatic brain injury: pathogenetic mechanisms. *Neuropathol Appl Neurobiol* **24,** 251-67.

Meng, F. and Lowell, C. A. (1998) A beta 1 integrin signaling pathway involving Src-family kinases, Cbl and PI-3 kinase is required for macrophage spreading and migration. *Embo J* **17,** 4391-403.

Milner, R. and Campbell, I. L. (2003) The extracellular matrix and cytokines regulate microglial integrin expression and activation. *J Immunol* **170,** 3850-8.

Mizutani, K. and Kimura, H. (1987) Immunohistochemical study of fibronectin imunoreactive structures demonstrated in response to brain injury of adult rats. *Acta Histochemica Cytochemica* **20,** 87-99.

Mograbi, B., Rochet, N., Emiliozzi, C. and Rossi, B. (1999) Adhesion of human monocytic THP-1 cells to endothelial cell adhesion molecules or extracellular matrix proteins via beta1 integrins regulates heparin binding epidermal growth factor-like growth factor (HB-EGF) expression. *Eur Cytokine Netw* **10,** 79-86.

Morales, D. M., Marklund, N., Lebold, D., Thompson, H. J., Pitkanen, A., Maxwell, W. L., Longhi, L., Laurer, H., Maegele, M., Neugebauer, E., Graham, D. I., Stocchetti, N. and McIntosh, T. K. (2005) Experimental models of traumatic brain injury: do we really need to build a better mousetrap? *Neuroscience* **136,** 971-89.

Morgan, M., Saba, S. and Gower, W. (2000) Fibronectin influences cellular proliferation and apoptosis similarly in LNCaP and PC-3 prostate cancer cell lines. **5,** 155-159.

Morganti-Kossmann, M. C., Rancan, M., Stahel, P. F. and Kossmann, T. (2002) Inflammatory response in acute traumatic brain injury: a double-edged sword. *Curr Opin Crit Care* **8,** 101-5.

Morris, R. (1984) Developments of a water-maze procedure for studying spatial learning in the rat. *J Neurosci Methods* **11,** 47-60.

Mosser, D. M. (1994) Receptors on phagocytic cells involved in microbial recognition. *Immunol Ser* **60,** 99-114.

Nag, S., Takahashi, J. L. and Kilty, D. W. (1997) Role of vascular endothelial growth factor in blood-brain barrier breakdown and angiogenesis in brain trauma. *J Neuropathol Exp Neurol* **56,** 912-21.

Nakamura, M., Raghupathi, R., Merry, D. E., Scherbel, U., Saatman, K. E. and McIntosh, T. K. (1999) Overexpression of Bcl-2 is neuroprotective after experimental brain injury in transgenic mice. *J Comp Neurol* **412,** 681-92.

Narayan, R. K., Michel, M. E., Ansell, B., Baethmann, A., Biegon, A., Bracken, M. B., Bullock, M. R., Choi, S. C., Clifton, G. L., Contant, C. F., Coplin, W. M., Dietrich, W. D., Ghajar, J., Grady, S. M., Grossman, R. G., Hall, E. D., Heetderks, W., Hovda, D. A., Jallo, J., Katz, R. L., Knoller, N., Kochanek, P. M., Maas, A. I., Majde, J., Marion, D. W., Marmarou, A., Marshall, L. F., McIntosh, T. K., Miller, E., Mohberg, N., Muizelaar, J. P., Pitts, L. H., Quinn, P., Riesenfeld, G., Robertson, C. S., Strauss, K. I., Teasdale, G., Temkin, N., Tuma, R., Wade, C., Walker, M. D., Weinrich, M., Whyte, J., Wilberger, J., Young, A. B. and Yurkewicz, L. (2002) Clinical trials in head injury. *J Neurotrauma* **19,** 503-57.

Nasu-Tada, K., Koizumi, S. and Inoue, K. (2005) Involvement of beta1 integrin in microglial chemotaxis and proliferation on fibronectin: different regulations by ADP through PKA. *Glia* **52,** 98-107.

Natale, J. E., Ahmed, F., Cernak, I., Stoica, B. and Faden, A. I. (2003) Gene expression profile changes are commonly modulated across models and species after traumatic brain injury. *J Neurotrauma* **20,** 907-27.

Newcomb, J. K., Zhao, X., Pike, B. R. and Hayes, R. L. (1999) Temporal profile of apoptotic-like changes in neurons and astrocytes following controlled cortical impact injury in the rat. *Exp Neurol* **158,** 76-88.

Owens, M. R. and Cimino, C. D. (1982) Synthesis of fibronectin by the isolated perfused rat liver. *Blood* **59,** 1305-9.

Pankov, R. and Yamada, K. M. (2002) Fibronectin at a glance. *J Cell Sci* **115,** 3861-3.

Park, K. I., Teng, Y. D. and Snyder, E. Y. (2002) The injured brain interacts reciprocally with neural stem cells supported by scaffolds to reconstitute lost tissue. *Nat Biotechnol* **20,** 1111-7.

Pasinetti, G. M., Nichols, N. R., Tocco, G., Morgan, T., Laping, N. and Finch, C. E. (1993) Transforming growth factor beta 1 and fibronectin messenger RNA in rat brain: responses to injury and cell-type localization. *Neuroscience* **54,** 893-907.

Pennell, N. A., Hurley, S. D. and Streit, W. J. (1994) Lectin staining of sheep microglia. *Histochemistry* **102,** 483-6.

Perris, R. and Perissinotto, D. (2000) Role of the extracellular matrix during neural crest cell migration. *Mech Dev* **95,** 3-21.

Perry, V. H., Anthony, D. C., Bolton, S. J. and Brown, H. C. (1997) The blood-brain barrier and the inflammatory response. *Mol Med Today* **3,** 335-41.

Pinkstaff, J. K., Detterich, J., Lynch, G. and Gall, C. (1999) Integrin subunit gene expression is regionally differentiated in adult brain. *J Neurosci* **19,** 1541-56.

Proctor, R. A. (1987) Fibronectin: an enhancer of phagocyte function. *Rev Infect Dis* **9 Suppl 4,** S412-9.

Properzi, F., Asher, R. A. and Fawcett, J. W. (2003) Chondroitin sulphate proteoglycans in the central nervous system: changes and synthesis after injury. *Biochem Soc Trans* **31,** 335-6.

Raghupathi, R., Conti, A. C., Graham, D. I., Krajewski, S., Reed, J. C., Grady, M. S., Trojanowski, J. Q. and McIntosh, T. K. (2002) Mild traumatic brain injury induces apoptotic cell death in the cortex that is preceded by decreases in cellular Bcl-2 immunoreactivity. *Neuroscience* **110,** 605-16.

Ramaswamy, S., Goings, G. E., Soderstrom, K. E., Szele, F. G. and Kozlowski, D. A. (2005) Cellular proliferation and migration following a controlled cortical impact in the mouse. *Brain Res* **1053,** 38-53.

Reese, A. C., Doran, J. E., Callaway, B. D. and Mansberger, A. R. (1982) Sequestration of fibronectin at the site of an injury. *Adv Shock Res* **8,** 119-27.

Reichardt, L. F. and Tomaselli, K. J. (1991) Extracellular matrix molecules and their receptors: functions in neural development. *Annu Rev Neurosci* **14,** 531-70.

Rietze, R. L., Valcanis, H., Brooker, G. F., Thomas, T., Voss, A. K. and Bartlett, P. F. (2001) Purification of a pluripotent neural stem cell from the adult mouse brain. *Nature* **412,** 736-9.

Risau, W. and Wolburg, H. (1990) Development of the blood-brain barrier. *Trends Neurosci* **13,** 174-8.

Roberts, I., Schierhout, G. and Alderson, P. (1998) Absence of evidence for the effectiveness of five interventions routinely used in the intensive care management of severe head injury: a systematic review. *J Neurol Neurosurg Psychiatry* **65,** 729-33.

Rosen, G. D. and Harry, J. D. (1990) Brain volume estimation from serial section measurements: a comparison of methodologies. *J Neurosci Methods* **35,** 115-24.

Sahuquillo, J. and Arikan, F. (2006) Decompressive craniectomy for the treatment of refractory high intracranial pressure in traumatic brain injury. *Cochrane Database Syst Rev***,** CD003983.

Sakai, T., Johnson, K. J., Murozono, M., Sakai, K., Magnuson, M. A., Wieloch, T., Cronberg, T., Isshiki, A., Erickson, H. P. and Fassler, R. (2001) Plasma fibronectin supports neuronal survival and reduces brain injury following transient focal cerebral ischemia but is not essential for skin-wound healing and hemostasis. *Nat Med* **7,** 324-30.

Saporta, S., Borlongan, C. V. and Sanberg, P. R. (1999) Neural transplantation of human neuroteratocarcinoma (hNT) neurons into ischemic rats. A quantitative dose-response analysis of cell survival and behavioral recovery. *Neuroscience* **91,** 519-25.

Scherbel, U., Raghupathi, R., Nakamura, M., Saatman, K. E., Trojanowski, J. Q., Neugebauer, E., Marino, M. W. and McIntosh, T. K. (1999) Differential acute and chronic responses of tumor necrosis factor-deficient mice to experimental brain injury. *Proc Natl Acad Sci U S A* **96,** 8721-6.

Schmidt, O. I., Heyde, C. E., Ertel, W. and Stahel, P. F. (2005) Closed head injury--an inflammatory disease? *Brain Res Brain Res Rev* **48,** 388-99.

Schouten, J. W., Fulp, C. T., Royo, N. C., Saatman, K. E., Watson, D. J., Snyder, E. Y., Trojanowski, J. Q., Prockop, D. J., Maas, A. I. and McIntosh, T. K. (2004) A review and rationale for the use of cellular transplantation as a therapeutic strategy for traumatic brain injury. *J Neurotrauma* **21,** 1501-38.

Schwartz, M. (2000) Autoimmune involvement in CNS trauma is beneficial if well controlled. *Prog Brain Res* **128,** 259-63.

Seledtsov, V. I., Rabinovich, S. S., Parlyuk, O. V., Kafanova, M. Y., Astrakov, S. V., Seledtsova, G. V., Samarin, D. M. and Poveschenko, O. V. (2005) Cell transplantation therapy in re-animating severely head-injured patients. *Biomed Pharmacother* **59,** 415-20.

Shear, D. A., Galani, R., Hoffman, S. W. and Stein, D. G. (2002) Progesterone protects against necrotic damage and behavioral abnormalities caused by traumatic brain injury. *Exp Neurol* **178,** 59-67.

Shear, D. A., Tate, M. C., Archer, D. R., Hoffman, S. W., Hulce, V. D., Laplaca, M. C. and Stein, D. G. (2004) Neural progenitor cell transplants promote long-term functional recovery after traumatic brain injury. *Brain Res* **1026,** 11-22.

Sheppard, A. M., Brunstrom, J. E., Thornton, T. N., Gerfen, R. W., Broekelmann, T. J., McDonald, J. A. and Pearlman, A. L. (1995) Neuronal production of fibronectin in the cerebral cortex during migration and layer formation is unique to specific cortical domains. *Dev Biol* **172,** 504-18.

Shinji, H., Seki, K., Tajima, A., Uchida, A. and Masuda, S. (2003) Fibronectin bound to the surface of Staphylococcus aureus induces association of very late antigen 5 and intracellular signaling factors with macrophage cytoskeleton. *Infect Immun* **71,** 140-6.

Sinson, G., Perri, B. R., Trojanowski, J. Q., Flamm, E. S. and McIntosh, T. K. (1997) Improvement of cognitive deficits and decreased cholinergic neuronal cell loss and apoptotic cell death following neurotrophin infusion after experimental traumatic brain injury. *J Neurosurg* **86,** 511-8.

Sinson, G., Voddi, M. and McIntosh, T. K. (1996) Combined fetal neural transplantation and nerve growth factor infusion: effects on neurological outcome following fluid-percussion brain injury in the rat. *J Neurosurg* **84,** 655-62.

Sonnenberg, A. (1993) Integrins and their ligands. *Curr Top Microbiol Immunol* **184,** 7-35.

Stabenfeldt, S. E., Garcia, A. J. and Laplaca, M. C. (2006) Thermoreversible laminin-functionalized hydrogel for neural tissue engineering. *J Biomed Mater Res A*.

Stein, D. G. (1991) Fetal brain tissue grafting as therapy for brain dysfunctions: Unanswered questions, unknown factors, and practical concerns. *J Neurosurg Anesthesiol* **3,** 170-89.

Stein, D. G. and Glasier, M. M. (1995) Some Practical and Theoretical Issues Concerning Fetal Brain- Tissue Grafts As Therapy For Brain Dysfunctions. *Behavioral and Brain Sciences* **18,** 36-45.

Sterio, D. C. (1984) The unbiased estimation of number and sizes of arbitrary particles using the disector. *J Microsc* **134 (Pt 2),** 127-36.

Subramanian, T. (2001) Cell transplantation for the treatment of Parkinson's disease. *Semin Neurol* **21,** 103-15.

Suzuki, M. and Choi, B. H. (1990) The behavior of the extracellular matrix and the basal lamina during the repair of cryogenic injury in the adult rat cerebral cortex. *Acta Neuropathol (Berl)* **80,** 355-61.

Suzuki, N., Yokoyama, F. and Nomizu, M. (2005) Functional sites in the laminin alpha chains. *Connect Tissue Res* **46,** 142-52.

Szabo, A. and Kalman, M. (2004) Disappearance of the post-lesional laminin immunopositivity of brain vessels is parallel with the formation of gliovascular junctions and common basal lamina. A double-labelling immunohistochemical study. *Neuropathol Appl Neurobiol* **30,** 169-77.

Takeuchi, M., Kageyama, S., Suzuki, H., Wada, T., Notsu, Y. and Ishii, F. (2003) Rheological properties of reversible thermo-setting in situ gelling solutions with the methylcellulose-polyethylene glycol-citric acid ternary system. *Colloid and Polymer Science* **281,** 1178-1183.

Tate, C. C., Tate, M. C. and LaPlaca, M. C. (Submitted 2006) Fibronectin and laminin increase in the mouse brain following controlled coritcal impact injury.

Tate, M. C., Garcia, A. J., Keselowsky, B. G., Schumm, M. A., Archer, D. R. and LaPlaca, M. C. (2004) Specific beta1 integrins mediate adhesion, migration, and differentiation of neural progenitors derived from the embryonic striatum. *Mol Cell Neurosci* **27,** 22-31.

Tate, M. C., Shear, D. A., Hoffman, S. W., Stein, D. G., Archer, D. R. and LaPlaca, M. C. (2002) Fibronectin promotes survival and migration of primary neural stem cells transplanted into the traumatically injured mouse brain. *Cell Transplant* **11,** 283-95.

Testaz, S. and Duband, J. L. (2001) Central role of the alpha4beta1 integrin in the coordination of avian truncal neural crest cell adhesion, migration, and survival. *Dev Dyn* **222,** 127-40.

Thurman, D. J., Alverson, C., Dunn, K. A., Guerrero, J. and Sniezek, J. E. (1999) Traumatic brain injury in the United States: A public health perspective. *J Head Trauma Rehabil* **14,** 602-15.

Tian, M., Hagg, T., Denisova, N., Knusel, B., Engvall, E. and Jucker, M. (1997) Laminin-alpha2 chain-like antigens in CNS dendritic spines. *Brain Res* **764,** 28-38.

Tonnesen, M. G., Feng, X. and Clark, R. A. (2000) Angiogenesis in wound healing. *J Investig Dermatol Symp Proc* **5,** 40-6.

Tsuru, A., Mizuguchi, M., Uyemura, K. and Takashima, S. (1996) Immunohistochemical expression of cell adhesion molecule L1 during development of the human brain. *Early Hum Dev* **45,** 93-101.

Verma, A. (2000) Opportunities for neuroprotection in traumatic brain injury. *J Head Trauma Rehabil* **15,** 1149-61.

Wagner, S. and Gardner, H. (2000) Modes of regulation of laminin-5 production by rat astrocytes. *Neurosci Lett* **284,** 105-8.

Willis, C., Lybrand, S. and Bellamy, N. (2004) Excitatory amino acid inhibitors for traumatic brain injury. *Cochrane Database Syst Rev***,** CD003986.

Winn, S. R., Lindner, M. D., Lee, A., Haggett, G., Francis, J. M. and Emerich, D. F. (1996) Polymer-encapsulated genetically modified cells continue to secrete human nerve growth factor for over one year in rat ventricles: behavioral and anatomical consequences. *Exp Neurol* **140,** 126-38.

Yanaka, K., Camarata, P. J., Spellman, S. R., McCarthy, J. B., Furcht, L. T., Low, W. C. and Heros, R. C. (1996) Synthetic fibronectin peptides and ischemic brain injury after transient middle cerebral artery occlusion in rats. *J Neurosurg* **85,** 125-30.

Yanaka, K., Camarata, P. J., Spellman, S. R., Skubitz, A. P., Furcht, L. T. and Low, W. C. (1997) Laminin peptide ameliorates brain injury by inhibiting leukocyte accumulation in a rat model of transient focal cerebral ischemia. *J Cereb Blood Flow Metab* **17,** 605-11.

Yandava, B. D., Billinghurst, L. L. and Snyder, E. Y. (1999) "Global" cell replacement is feasible via neural stem cell transplantation: evidence from the dysmyelinated shiverer mouse brain. *Proc Natl Acad Sci U S A* **96,** 7029-34.

Zhang, Z., Vuori, K., Reed, J. C. and Ruoslahti, E. (1995) The alpha 5 beta 1 integrin supports survival of cells on fibronectin and up-regulates Bcl-2 expression. *Proc Natl Acad Sci U S A* **92,** 6161-5.

Zhao, L. R., Spellman, S., Kim, J., Duan, W. M., McCarthy, J. B. and Low, W. C. (2005) Synthetic fibronectin peptide exerts neuroprotective effects on transient focal brain ischemia in rats. *Brain Res* **1054,** 1-8.